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(54) Title: METHODS AND COMPOSITIONS FOR GENE THERAPY FOR THE TREATMENT OF DEFECTS IN LIPOPROTEIN METABOLISM (57) Abstract The invention provides a recombinant viral vector comprising the DNA of, or corresponding to, at least a portion of the genome of an adenovirus, which portion is capable of infecting a hepatic cell; and a human <i>VLDL</i> receptor gene operatively linked to regulatory sequences directing its expression. The vector is capable of expressing the normal <i>VLDL</i> receptor gene product in hepatic cells <i>in vivo</i> or <i>in vitro</i> . This viral vector is useful in the treatment of metabolic disorders caused by the accumulation of <i>LDL</i> in plasma, such as familial hypercholesterolemia or familial combined hyperlipidemia.		

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METHODS AND COMPOSITIONS FOR GENE THERAPY FOR THE TREATMENT OF DEFECTS IN LIPOPROTEIN METABOLISM

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5 The United States government has rights in this
invention.

Field of the Invention

The present invention relates to the field of
somatic gene therapy and the treatment of genetic
10 disorders related to lipoprotein metabolism.

Background of the Invention

The metabolism of lipids, particularly
cholesterol, involves the interaction of a number of
lipoproteins and apolipoproteins. Very low density
15 lipoprotein (VLDL) and apolipoprotein E (apoE) are key
precursor molecules in the production of low density
lipoprotein (LDL) and in the overall metabolism of
lipids, including cholesterol. LDL is the major
cholesterol-transport lipoprotein in human plasma.

20 The VLDL/apoE receptors are expressed in heart,
skeletal muscle, and adipose tissue [F. M. Wittmaack et
al, Endocrinol., 136(1):340-348 (1995)] with lower levels
of expression in the kidney, placenta, pancreas, and
brain. This receptor has been suggested to play a role
25 in the uptake of triglyceride-rich lipoprotein particles
by specific organs. The cDNA encoding the putative human
VLDL receptor was recently cloned [M. E. Gafvels et al,
Som. Cell Mol. Genet., 19:557-569 (1993)], incorporated by
reference herein]. The receptor for LDL is located in
30 coated pits on the surfaces of cells in the liver and
other organs.

As depicted in Fig. 1A, in a normal healthy
human, the molecules apolipoprotein B48 (Apo-B48),
apolipoprotein C-II (Apo-C-II) and Apo E form a

chylomicron particle in plasma passing through the intestines, which interacts with a chylomicron remnant receptor in the liver. After metabolism of the chylomicrons taken up by the remnant receptor, the liver produces the primary lipoprotein, VLDL, which contains Apo-E, Apo-C-II and apolipoprotein B100 (Apo B100). VLDL is metabolized into LDL, which binds to the LDL receptor in the liver via Apo B100. The LDL receptor in the liver facilitates the uptake of LDL by receptor-mediated endocytosis. LDL is degraded in lysosomes, and its cholesterol is released for metabolic use.

Defects in the metabolism of such lipoproteins and/or receptors result in several serious metabolic disorders. The human disease familial hypercholesterolemia (FH) is caused primarily by one or more mutations in the gene encoding the LDL receptor. FH is characterized clinically by (1) an elevated concentration of LDL; (2) deposition of LDL-derived cholesterol in tendons and skin (xanthomas) and in arteries (atheromas); and (3) inheritance as an autosomal dominant trait with a gene dosage effect. Individuals with FH develop premature coronary heart disease, usually in childhood. Heterozygotes number about 1 in 500 persons, placing FH among the most common inborn errors of metabolism. Heterozygotes have twofold elevations in plasma cholesterol (350 to 550 mg/dl) from birth and tend to develop tendon xanthomas and coronary atherosclerosis after age 20. Homozygotes number 1 in 1 million persons and are characterized by severe hypercholesterolemia (650 to 1000 mg/dl), cutaneous xanthomas which appear within the first 4 years of life, and coronary heart disease which begins in childhood and frequently causes death before age 20. [J. Goldstein et al, "Familial Hypercholesterolemia", Chapter 48, in The Metabolic Basis of Inherited Disease, 6th ed., C. R. Scriver et al

(eds), McGraw-Hill Information Services Co., NY, NY, (1989) pp. 1215-1250].

Another metabolic disorder is familial combined hyperlipidemia (FCH) which was first associated with hyperlipidemia in survivors of myocardial infarction and their relatives. FCH patients generally have one of three phenotypes: (1) elevated levels of VLDL, (2) elevated levels of LDL, or (3) increases in the levels of both lipoproteins in plasma. Unlike FH, FCH appears in only 10 to 20 percent of patients in childhood, usually in the form of hypertriglyceridemia. Homozygosity for the trait may result in severe hypertriglyceridemia. [J. Goldstein et al, "Disorders of the Biogenesis and Secretion of Lipoproteins", Chapter 44B in The Metabolic Basis of Inherited Disease, 6th ed., C. R. Scriver et al (eds), McGraw-Hill Information Services Co., NY, NY, (1989) pp. 1155-1156]. This disorder is also associated with the appearance of glucose intolerance and obesity in a number of individuals.

The most striking abnormality of FCH is marked elevation of VLDL content of plasma. Increased production of VLDL leads to an expanded plasma pool of VLDL in some individuals, but in others with more efficient lipolysis, it results in increased levels of LDL. FCH is characterized by an excess production of LDL, rather than a genetic defect in the LDL receptor. The LDL receptors of cultured fibroblasts appear to be normal in FCH patients.

Clinical experience suggests that FCH is at least five times as prevalent as FH, occurring in about 1 percent of the North American population. The predilection toward coronary artery disease among patients with this disorder makes it the most prominent known metabolic cause of premature atherosclerosis [J. Goldstein et al, cited above].

When *LDL* receptors are deficient as in FH (see Fig. 1B), or excess *LDL* is produced due to excess *VLDL* as in FCH, the efficient removal of *LDL* from plasma by the liver declines, and the level of *LDL* rises in inverse proportion to the receptor number. The excess plasma *LDL* is deposited in connective tissues and in scavenger cells, resulting in the symptoms of either disorder.

Presently, treatment for FH and FCH is directed at lowering the plasma level of *LDL* by the administration of drugs, i.e., combined administration of a bile acid-binding resin and an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase for treatment of FH and niacin for treatment of FCH. However, FH homozygotes with two nonfunctional genes are resistant to drugs that work by stimulating *LDL* receptors. Similarly, such drugs are not particularly effective in FCH. In FH homozygotes, plasma *LDL* levels can be lowered only by physical or surgical means.

Administration of normal *LDL* receptor genes by gene therapy using an adenovirus vector has been contemplated for the treatment of FH. Adenovirus vectors are capable of providing extremely high levels of transgene delivery to virtually all cell types, regardless of the mitotic state. The efficacy of this system in delivering a therapeutic transgene in vivo that complements a genetic imbalance has been demonstrated in animal models of various disorders [K. F. Kozarsky et al, Somatic Cell Mol. Genet., 19:449-458 (1993) ("Kozarsky I"); K. F. Kozarsky et al, J. Biol. Chem., 269:13695-13702 (1994) ("Kozarsky II"); Y. Watanabe, Atherosclerosis, 36:261-268 (1986); K. Tanzawa et al, FEBS Letters, 118(1):81-84 (1980); J.L. Golasten et al, New Engl. J. Med., 309:288-296 (1983); S. Ishibashi et al, J. Clin. Invest., 92:883-893 (1993); and S. Ishibashi et al, J. Clin. Invest., 93:1885-1893 (1994)]. The use

of adenovirus vectors in the transduction of genes into hepatocytes *in vivo* has previously been demonstrated in rodents and rabbits [see, e.g., Kozarsky II, cited above, and S. Ishibashi et al, J. Clin. Invest., 92:883-893 (1993)].

Recent research has shown that introduction of a recombinant adenovirus encoding the human *LDL* receptor ("*LDLR*") cDNA into the livers of *LDL* receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits, which mimic the condition of FH, resulted in large, transient reductions in plasma cholesterol. The transient nature of the effect of recombinant adenoviruses in most situations is attributed to the development of cellular immune responses to the virus-infected cells and their subsequent elimination. Antigenic targets for immune mediated clearance are viral proteins expressed from the recombinant viral genome and/or the product of the transgene, which in this case, is the *LDL* receptor protein [Y. Yang et al, Proc. Natl. Acad. Sci., USA, 91:4407-4411 (May 1994); Y. Yang et al, Immun., 1:433-442 (August 1994)].

Additionally, repeated reinfusions of the *LDLR* gene-containing adenovirus did not produce similar, subsequent cholesterol reductions due to the development of neutralizing anti-adenovirus antibodies [Kozarsky I and Kozarsky II, cited above; see also Y. Yang et al, Immun., 1:433-442 (August 1994), all incorporated by reference herein].

There remains a need in the art for therapeutic compositions and gene therapy strategies which enable effective treatment and/or prevention of FH and FCH, as well as other defects in lipoprotein metabolism.

Summary of the Invention

In one aspect, the invention provides a recombinant viral vector comprising the DNA of, or corresponding to, at least a portion of the genome of an adenovirus, which portion is capable of infecting a hepatic cell; and a human VLDL receptor ("VLDLR") gene operatively linked to regulatory sequences directing its expression, the vector capable of expressing the VLDLR gene product in the hepatic cell *in vivo* or *in vitro*.

In another aspect, the invention provides a mammalian cell infected with the viral vector described above.

In still a further aspect, the invention provides a method for delivering and stably integrating a VLDLR gene into the chromosome of a mammalian hepatocyte cell comprising introducing into said cell an effective amount of a recombinant viral vector described above.

Another aspect of this invention is a method for treating a patient having a metabolic disorder comprising administering to the patient by an appropriate route an effective amount of an above described vector containing a normal VLDLR gene, wherein said VLDLR gene is integrated into the chromosome of said patient's hepatocytes and said receptor is expressed stably *in vivo* at a location in the body where it is not normally expressed.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1A is a schematic drawing of normal human and rabbit lipoprotein metabolism. The apolipoproteins are referred to as B48, B100, C-II, and E. LDL and VLDL are identified.

Fig. 1B is a schematic drawing of lipoprotein metabolism in FH patients and WHHL rabbits. The abbreviations are as described in Fig. 1A.

Fig. 1C is a schematic drawing of lipoprotein metabolism in rabbits infused with the recombinant *VLDLR* gene according to the invention.

Fig. 2 is a schematic drawing of plasmid pAd.CMV*VLDLR*, which contains adenovirus map units 0-1 (Ad 0-1), followed by a cytomegalovirus enhancer/promoter (CMV enh/prom), a human *VLDLR* gene, a polyadenylation signal (pA), adenovirus map units 9-16 (Ad 9-16) and plasmid sequences from plasmid pAT153 including an origin of replication and ampicillin resistance gene. Restriction endonuclease enzymes are represented by conventional designations in the plasmid construct.

Fig. 3 is a schematic map of recombinant adenovirus H5.010CMV*VLDLR*, in which 0 to 100 represent the map units of an adenovirus type 5 (Genbank Accession No. M73260), and the CMV/*VLDLR*/pA minicassette of pAd.CMV*VLDLR* is inserted between adenovirus map units 1 and 9, with the remaining Ad5 map units 9-100 having a partial E3 gene deletion between about map unit 78.5 and about 84.3.

Fig. 4A is a graph plotting changes in plasma cholesterol levels in mg/dl for WHHL rabbits as a function of days before and after receiving recombinant adenovirus H5.010CMVlacZ. The symbols represent individual animals. See Example 3.

Fig. 4B is a graph plotting changes in plasma cholesterol levels in mg/dl for WHHL rabbits as a function of days before and after receiving recombinant adenovirus H5.010CMV*VLDLR*. The symbols represent the response of four individual animals. See Example 3.

Fig. 5 is a bar graph representing cholesterol levels (measured as % pre-infusion) in mice infused with recombinant adenovirus H5.010CMVlacZ (lacZ), recombinant adenovirus H5.010CMVVLDLR and recombinant adenovirus H5.010CBhLDLR. The dotted bars represent pre-infusion levels and the solid bars represent post-infusion levels. See Example 4.

Fig. 6 is a bar graph representing cholesterol levels, specifically the levels of the fractions of plasma lipoproteins (measured as mg/fraction) in mice infused with recombinant adenovirus H5.010CMVlacZ (lacZ), recombinant adenovirus H5.010CMVVLDLR and recombinant adenovirus H5.010CBhLDLR. The solid bars represent proteins or fragments falling within a density (d) > 1.21; the thickly cross-hatched bars represent HDL; the closely cross-hatched bars represent LDL, the spaced apart slanted hatched bars represent intermediate density lipoprotein (IDL), and the clear bars represent VLDL levels. See Example 4.

Fig. 7A is a graph plotting changes in cholesterol levels (measured in mg/dl) as a function of days pre- and post-infusion for mice infused with H5.010CMVlacZ. The symbols represent the responses of individual animals. See Example 5.

Fig. 7B is a graph plotting changes in cholesterol levels (measured in mg/dl) as a function of days pre- and post-infusion for mice infused with H5.010CBhLDLR. The symbols are the same as for Fig. 7A. See Example 5.

Fig. 7C is a graph plotting changes in cholesterol levels (measured in mg/dl) vs. days pre and post-infusion for mice infused with H5.010CMVVLDLR. The symbols are the same as for Fig. 7A. See Example 5.

Fig. 7D is a graph providing the average results \pm standard deviation from two experiments for mice infused with H5.010CMVLacZ (n=9) or with H5.010CMVVLDLR (n=10). Average pre-infusion cholesterol levels were 870 mg/dl and 946 mg/dl, respectively. Asterisks indicate $p < 0.05$.

Figs. 8A-8F are the DNA sequence [SEQ ID NO: 1] with encoded amino acid sequence [SEQ ID NO: 2] of the human VLDL receptor gene, as reported by Gafvels et al, cited above.

Figs. 9A-9I are the DNA sequence of pAd.CMVVLDLR [SEQ ID NO: 3], in which Ad 0-1 spans nucleotides 12-364, CMV ehv/prom spans nucleotides 381-862; nucleotides 966-4107 encode VLDLR, pA spans nucleotides 4192-4390; Ad 9.2-16.1 span nucleotides 4417-6880 and nucleotides 6881-9592 are pAT153 sequences.

Fig. 10A is a bar chart illustrating the CTL activity (average \pm standard deviation) measured at an effector:target cell ratio of 25:1. ** = $p < 0.005$; * = $p < 0.05$.

Fig. 10B is a line graph illustrating the CTL activity measured against varying effector:target ratios.

Fig. 11A is a graph summarizing neutralizing antibody titer present in BAL samples of C57BL/6 mice adenovirus-infected on day 0 and necrotized on day 28 as described in Example 9. Control represents normal mice ("control"); CD4 mAB represents CD4⁺ cell depleted mice; IL-12 represents IL-12 treated mice and IFN- γ represent IFN- γ treated mice.

Fig. 11B is a graph summarizing the relative amounts (OD₄₀₅) of IgG present in BAL samples. The symbols are as described in Fig. 11A.

Fig. 11C is a graph summarizing the relative amounts (OD₄₀₅) of IgA present in BAL samples. The symbols are as described in Fig. 11A.

Detailed Description of the Invention

The present invention provides novel compositions and methods which enable the therapeutic treatment of metabolic disorders, such as FH and FCH, characterized by the accumulation of *LDL* in human plasma. This invention provides for the use of a viral vector to introduce and stably express a gene normally expressed in mammals, i.e., the gene encoding a normal receptor for very low density lipoprotein (*VLDLR*), in a location in the body where that gene is not naturally present, i.e., in the liver.

The methods and compositions of the present invention overcome the problems previously identified in the gene therapy treatment of *LDL* receptor-deficient individuals. As described in detail below, by use of a viral vector capable of targeting cells of the liver, the *VLDLR* receptor gene is introduced into and stably expressed in liver cells. The present invention differs from direct gene replacement in that the *VLDLR* receptor protein is expressed normally in *LDL* receptor deficient individuals, e.g., the macrophages. Thus, gene therapy using a liver-directed viral vector carrying a *VLDLR* gene would result not in expression of a new gene product, but rather, in *de novo* expression in an organ which otherwise does not express the gene product. Importantly, the patient does not mount an immune response against the *VLDLR* gene product expressed in the liver because the vector-delivered *VLDLR* gene is not recognized as a foreign antigen, and there is no induction of CTL-mediated elimination of the transfected cell. In contrast, CTL-mediated elimination of viral vectors is a problem when an *LDLR* gene is administered to an *LDLR*-deficient individual with FH [see, e.g., Kozarsky I and II, cited above].

Due to this recognition of the *VLDLR* gene by the patient's immune system as a known gene, and to the tendency of hepatocytes to have a long life in circulation, the hepatocytes transfected with the vector of this invention, which express the *VLDLR* gene, tend to be stable and *VLDLR* expression is not transient. *VLDLR* gene expression in transfected hepatocytes occurs for the duration of the hepatocyte's life. The lipoprotein metabolic disorder may be treated for longer times without the need for reinfusing the viral vector, thus limiting the number of viral exposures and potential immune reactions to vector-encoded viral proteins.

The vectors and methods of this invention can provide gene therapy useful to treat and/or supplement current treatments for lipoprotein metabolic disorders. The presence of the *VLDL* receptor gene in the transfected hepatocytes according to this invention permits the binding of *VLDL*, a precursor of *LDL*, from the plasma at the site of the liver, thereby decreasing the amount of *VLDL* in plasma. The decrease in *VLDL* in the plasma consequently decreases the production of plasma *LDL*.

For example, in FH, this reduction in plasma *LDL* can compensate for the defective *LDL* receptors in the liver. In FCH, this reduced production of plasma *LDL* from *VLDL* prevents the normal *LDL* receptors in the liver from becoming overloaded by excess *LDL*, and reduces the excess *VLDL* which contributes to the disorder. Compare, for example, the schematic representations of the normal operation of lipid metabolism (Fig. 1A) to the abnormal metabolism caused by FH (Fig. 1B) and then to the method of this invention (Fig. 1C).

I. Recombinant Viral Particles as Gene Therapy Vectors

The compositions of this invention involve the construction of desirable gene therapy vectors, which are capable of delivering and stably integrating a functional, normal *VLDL* receptor gene to hepatocytes. Such gene therapy vectors include a selected virus vector, desirably deleted in one or more viral genes, a minigene containing the *VLDLR* gene under the control of regulatory sequences, and optional helper viruses and/or packaging cell lines which supply to the viral vectors any necessary products of deleted viral genes.

The viral sequences used in the vectors, helper viruses, if needed, and recombinant viral particles, and other vector components and sequences employed in the construction of the vectors described herein are obtained from commercial or academic sources based on previously published and described sequences. These viral materials may also be obtained from an individual patient. The viral sequences and vector components may be generated by resort to the teachings and references contained herein, coupled with standard recombinant molecular cloning techniques known and practiced by those skilled in the art. Modifications of existing nucleic acid sequences forming the vectors, including sequence deletions, insertions, and other mutations taught by this specification may be generated using standard techniques.

The methods employed for the selection of viral sequences useful in a vector, the cloning and construction of *VLDLR* "minigene" and its insertion into a desired viral vector and the production of a recombinant infectious viral particle by use of helper viruses and the like are within the skill in the art given the teachings provided herein.

A. Construction of the "Minigene"

By "minigene" is meant the combination of the *VLDLR* gene and the other regulatory elements necessary to transcribe the gene and express the gene product *in vivo*. The human *VLDL* receptor sequence has been provided [see, Gafvels et al, cited above; SEQ ID NOS: 1 and 2]. Generally, the entire coding region of this receptor sequence is used in the minigene; the 5' and 3' untranslated sequences of SEQ ID NO: 1 are not essential to the minigene. *VLDL* receptor genes of other mammalian origins, e.g., rabbit, monkey, etc., may also be useful in this invention.

The *VLDL* receptor gene (*VLDLR*) is operatively linked to regulatory components in a manner which permits its transcription. Such components include conventional regulatory elements necessary to drive expression of the *VLDLR* transgene in a cell transfected with the viral vector. Thus the minigene also contains a selected promoter which is linked to the transgene and located, with other regulatory elements, within the selected viral sequences of the recombinant vector.

Selection of the promoter is a routine matter and is not a limitation of this invention. Useful promoters may be constitutive promoters or regulated (inducible) promoters, which will enable control of the amount of the transgene to be expressed. For example, a desirable promoter is that of the cytomegalovirus immediate early promoter/enhancer [see, e.g., Boshart et al, *Cell*, 41:521-530 (1985)]. Another desirable promoter includes the Rous sarcoma virus LTR promoter/enhancer. Still another promoter/enhancer sequence is the chicken cytoplasmic β -actin promoter [T. A. Kost et al, *Nucl. Acids Res.*, 11(23):8287 (1983)]. Other suitable promoters may be selected by one of skill in the art.

The minigene may also desirably contain nucleic acid sequences heterologous to the viral vector sequences including sequences providing signals required for efficient polyadenylation of the transcript (poly-A or pA) and introns with functional splice donor and acceptor sites. A common poly-A sequence which is employed in the exemplary vectors of this invention is that derived from the papovavirus SV-40. The poly-A sequence generally is inserted in the minigene following the transgene sequences and before the viral vector sequences. A common intron sequence is also derived from SV-40, and is referred to as the SV-40 T intron sequence. A minigene of the present invention may also contain such an intron, desirably located between the promoter/enhancer sequence and the transgene. Selection of these and other common vector elements are conventional [see, e.g., Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d ed., Cold Spring Harbor Laboratory, New York (1989) and references cited therein] and many such sequences are available from commercial and industrial sources as well as from Genbank.

As stated above, the minigene is located in the site of any selected deletion in the viral vector. See Example 1 below.

B. Construction of The Viral Plasmid Vector

Although a number of viral vectors have been suggested for gene therapy, the most desirable vector for this purpose is a recombinant adenoviral vector or adeno-associated vector. Adenovirus vectors as described below are preferred because they can be purified in large quantities and highly concentrated, and the virus can transduce genes into non-dividing cells.

However, it is within the skill of the art for other adenovirus, or even retrovirus, vaccinia or other virus vectors to be similarly constructed.

Adenoviruses are eukaryotic DNA viruses
5 that can be modified to efficiently deliver a therapeutic or reporter transgene to a variety of cell types. Human adenoviruses comprise a linear, approximately 36 kb double-stranded DNA genome, which is divided into 100 map units (m.u.), each of which is 360 bp in length. The DNA
10 contains short inverted terminal repeats (ITR) at each end of the genome that are required for viral DNA replication. The gene products are organized into early (E1 through E4) and late (L1 through L5) regions, based on expression before or after the initiation of viral DNA
15 synthesis [see, e.g., Horwitz, Virology, 2d edit., ed. B. N. Fields, Raven Press, Ltd. , New York (1990)]. The general adenoviruses types 2 and 5 (Ad2 and Ad5, respectively), are not associated with human malignancies.

20 Suitable adenovirus vectors useful in gene therapy are well known [see, e.g., M. S. Horwitz et al, "Adenoviridae and Their Replication", Virology, second edition, pp. 1712, ed. B. N. Fields et al, Raven Press Ltd., New York (1990); M. Rosenfeld et al, Cell, 68:143-
25 155 (1992); J. F. Engelhardt et al, Human Genet. Ther., 4:759-769 (1993); Y. Yang et al, Nature Genet., 7:362-269 (1994); J. Wilson, Nature, 365:691-692 (Oct. 1993); B. J. Carter, in "Handbook of Parvoviruses", ed. P. Tijsser, CRC Press, pp. 155-168 (1990). The selection of the
30 adenovirus type is not anticipated to limit the following invention.

Adenovirus vectors useful in this invention may include the DNA sequences of a number of adenovirus types. The adenovirus sequences useful in the
35 vectors described herein may be obtained from any known

adenovirus type, including the presently identified 41 human types [see, e.g., Horwitz, cited above]. The sequence of a strain of adenovirus type 5 may be readily obtained from Genbank Accession No. M73260. Similarly, adenoviruses known to infect other animals may also be employed in the vector constructs of this invention. A variety of adenovirus strains are available from the American Type Culture Collection, Rockville, Maryland, or available by request from a variety of commercial and institutional sources.

Adenovirus vectors useful in this invention include recombinant, defective adenoviruses, optionally bearing other mutations, e.g., temperature-sensitive mutations, deletions and hybrid vectors formed with adenovirus/adeno-associated virus sequences. Suitable vectors are described in the published literature [see, for example, Kozarsky I and II, cited above, and references cited therein, U. S. Patent No. 5,240,846 and the co-pending applications incorporated herein by reference below.

Useful adenovirus vectors for delivery of the VLDLR gene to the liver, minimal adenovirus nucleic acid sequences may be used to make a vector, in which case the use of a helper virus to produce a hybrid virus particle is required. Alternatively, only selected deletions of one or more adenovirus genes may be employed to construct a viral vector. Deleted gene products can be supplied by using a selected packaging cell line which supplies the missing gene product.

1. Recombinant Minimal Adenovirus

Desirable adenovirus (Ad) vectors useful in the present invention are described in detail in co-pending, co-owned U.S. Patent Application Serial No. 08/331,381, which is incorporated by reference herein for the purpose of describing these vectors.

Briefly summarized, the minimal Ad virus is a viral particle containing only the adenovirus cis-elements necessary for replication and virion encapsidation, but otherwise deleted of all adenovirus genes. That is, the vector contains only the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as origins of replication) and the native 5' packaging/enhancer domain, that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter. This left terminal (5') sequence of the Ad5 genome spans bp 1 to about 360 of the conventional published Ad5 adenovirus genome, also referred to as map units 0-1 of the viral genome, and generally is from about 353 to about 360 nucleotides in length. This sequence includes the 5'ITR (bp 1 to about 103 of the adenovirus genome); and the packaging/enhancer domain (bp about 194 to about 358 of the adenovirus genome). The minimal 3' adenovirus sequences of the adenovirus vector may include the right terminal (3') ITR sequence of the adenoviral genome spanning about bp 35,353 to the end of the adenovirus genome, or map units -98.4-100. This sequence is generally about 580 nucleotide in length. Between such sequences, a *VLDLR* minigene, as described above, is inserted.

Production of an infectious particle from this minimal Ad viral vector involves the assistance of a helper virus, as discussed below. A second type of minimal vector also disclosed in the above-incorporated reference places the 5' Ad terminal sequence in a head-to-tail arrangement relative to the 3' terminal sequence. The minimal Ad vector co-infected with a helper virus and/or a packaging cell line provides all of the viral gene products necessary to produce an infective recombinant viral particle containing the *VLDLR* minigene.

Alternatively, this vector can contain additional adenovirus gene sequences, which then are not required to be supplied by a helper virus.

2. Other Defective Adenoviruses

5 Recombinant, replication-deficient adenoviruses useful for gene therapy of this invention may be characterized by containing more than the minimal adenovirus sequences defined above. These other Ad
10 vectors can be characterized by deletions of various portions of gene regions of the virus, and infectious virus particles formed by the optional use of helper
15 viruses and/or packaging cell lines. Suitable defective adenoviruses are described in more detail in Kozarsky and Wilson, Curr. Opin. Genet. Devel., 3:499-503 (1993);
Kozarsky I and II, cited above, and references cited therein, all incorporated herein by reference.

 As one example, suitable vectors may be formed by deleting all or a sufficient portion of the adenoviral early immediate early gene E1a (which spans mu
20 1.3 to 4.5) and delayed early gene E1b (which spans mu 4.6 to 11.2) so as to eliminate their normal biological functions. These replication-defective E1-deleted
25 viruses are capable of replicating and producing infectious virus when grown on an adenovirus-transformed, complementation human embryonic kidney cell line, the 293
30 cell [ATCC CRL1573], containing functional adenovirus E1a and E1b genes which provide the corresponding gene products in trans. The resulting virus is capable of infecting many cell types and can express a transgene
(i.e., *VLDLR* gene), but cannot replicate in most cells that do not carry the E1 region DNA unless the cell is infected at a very high multiplicity of infection. Extensive experience in animals indicates that E1-deleted vectors are not particularly desirable for gene therapy

because low levels of viral proteins are expressed which elicit destructive cellular immune responses.

As a preferred example, all or a portion of the adenovirus delayed early gene E3 (which spans mu 76.6 to 86.2) may be eliminated from the adenovirus sequence which forms a part of the hybrid construct. The function of E3 is irrelevant to the function and production of the recombinant virus particle. For example, Ad vectors may be constructed with a therapeutic minigene inserted into the E1-deleted region of the known mutant Ad5 sub360 backbone [J. Logan et al, Proc. Natl. Acad. Sci. USA, 81:3655-3659 (1984)]; or the Ad5 mutant dl7001 backbone [Dr. William Wold, Washington University, St. Louis]. Both mutant viruses also contain a deletion in the E3 region of the adenoviral genome; in sub360, at 78.5 to 84.3 mu, and in dl7001, at 78.4 to 86 mu. The life cycle of both sub360 and dl7001 display wild type characteristics.

More preferred adenovirus vectors may be constructed having a deletion of the E1 gene, at least a portion of the E3 region, and an additional deletion within adenovirus genes other than E1 and E3 to accommodate the *VLDLR* minigene and/or other mutations which result in reduced expression of adenoviral protein and/or reduced viral replication. For example, all or a portion of the adenovirus delayed early gene E2a (which spans mu 67.9 to 61.5) may be eliminated from the adenovirus vector. It is also anticipated that portions of the other delayed early genes E2b (which spans mu 29 to 14.2) and E4 (which spans mu 96.8 to 91.3) may also be eliminated from the adenovirus vector.

Deletions may also be made in any of the late genes L1 through L5, which span mu 16.45 to 99 of the adenovirus genome. Similarly, deletions may be useful in the intermediate genes IX (which maps between

mu 9.8 and 11.2) and IVa₂ (which maps between 16.1 to 11.1). Other useful deletions may also be made in the other structural or non-structural adenovirus genes.

An adenovirus sequence for use in the present invention may contain deletions of E1 only. Alternatively, deletions of entire genes or portions effective to destroy their biological activity may be used in any combination. For example, in one exemplary vector, the adenovirus sequence may contain deletions of the E1 genes and the E3 gene, or of the E1, E2a and E3 genes, or of the E1 and E4 genes, or of E1, E2a and E4 genes, with or without deletion of E3, and so on.

Vectors may also contain additional mutations in genes necessary for viral replication. Adenovirus vectors may contain a mutation which produces temperature-sensitive (ts) viruses. Among such mutations include the incorporation of the missense temperature-sensitive mutation in the E2a region found in the Ad5 H5ts125 strain [P. Vander Vliet et al, J. Virol., 15:348-354 (1975)] at 62.5 mu. A single amino acid substitution (62.5 mu) at the carboxy end of the 72 kd protein (DBP) produced from the E2a gene in this strain produces a protein product which is a single-stranded DNA binding protein and is involved in the replication of adenoviral genomic DNA. At permissive temperatures (approximately 32°C) the ts strain is capable of full life cycle growth on HeLa cells, while at non-permissive temperatures (approximately 38°C), no replication of adenoviral DNA is seen. In addition, at non-permissive temperatures, decreased immunoreactive 72 kd protein is seen in HeLa cells.

Exemplary vectors for use in this invention, for example, may be obtained by combining

fragments from three independent DNA constructs, including sub360 or dl7001, H5ts125, and a cDNA plasmid with Ela sequences placed 5' to a therapeutic minigene. This type of vector is described, for example, by J. F. Engelhardt et al, Proc. Natl. Acad. Sci. USA, 91:6196-6200 (June 1994); Y. Yang et al, Nature Genet., 7: 362-369 (July, 1994) and references cited therein, all references incorporated herein by reference. Due to the mutations in the vector, there is reduced viral replication, reduction in expressed protein and an increase in the persistence of transgene expression. Other preferred adenovirus vectors contain the H5ts125 mutation in addition to E3 deletions of sub360 and dl7001. The minigene containing *VLDLR* as the transgene may be inserted into any deleted region of the selected Ad virus.

An exemplary Ad virus vector used to demonstrate this invention is the defective adenovirus vector H5.010CMVVLDLR, which contains adenovirus sequences Ad m.u. 0-1, followed by a *VLDLR* minigene, and the sequence Ad m.u.9 to 100 with small deletions in E3. See Fig. 3, described above. The recombinant adenovirus was fully deleted of Ela, Elb and partially deleted of E3. This recombinant virus vector is described in detail in Example 1.

3. Ad/AAV Hybrid Vectors

Another preferred vector is a hybrid Ad/AAV vector, which is the subject of co-owned, co-pending U.S. Patent Application Ser. No. 08/331,384, which is incorporated by reference herein.

At a minimum, the adenovirus nucleic acid sequences employed in the hybrid vector of this invention are the minimal adenovirus genomic sequences required for packaging adenoviral genomic DNA into a preformed capsid head, as described above. The entire

adenovirus 5' sequence containing the 5' ITR and packaging/enhancer region can be employed as the 5' adenovirus sequence in the hybrid vector. The 3' adenovirus sequences of the vector include the right terminal (3') ITR sequence of the adenoviral genome discussed above. Some modifications to these sequences which do not adversely affect their biological function may be acceptable.

Also part of the hybrid vectors of this invention are sequences of an adeno-associated virus. The AAV sequences useful in the hybrid vector are the viral sequences from which the *rep* and *cap* polypeptide encoding sequences are deleted. More specifically, the AAV sequences employed are the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences [See, e.g., B. J. Carter, cited above]. The AAV ITR sequences are about 143 bp in length. Substantially the entire sequences encoding the ITRs are used in the vectors, although some degree of minor modification of these sequences is expected to be permissible for this use. The ability to modify these ITR sequences is within the skill of the art. See, e.g., Sambrook et al, cited above.

In the Ad/AAV hybrid vector construct, the AAV sequences are flanked by the adenovirus sequences discussed above. The 5' and 3' AAV ITR sequences themselves flank a *VLDLR* minigene sequence as described above. Thus, the sequence formed by the *VLDLR* minigene and flanking 5' and 3' AAV sequences may be inserted at any deletion site in the adenovirus sequences of the vector. For example, the AAV sequences are desirably inserted at the site of deleted *E1a/E1b* genes of the adenovirus, i.e., after map unit 1. Alternatively, the AAV sequences may be inserted at an *E3* deletion, *E2a* deletion, and so on. If only the

adenovirus 5' ITR/packaging sequences and 3' ITR sequences are used in the vector, the AAV sequences are inserted between them.

As described above for the minimum
5 adenovirus sequences, those gene sequences not present in the adenovirus portion of the hybrid vector must be supplied by either a packaging cell line and/or a helper adenovirus to generate the recombinant hybrid viral particle. Uptake of this hybrid virus by the cell is
10 caused by the infective ability contributed to the vector by the adenovirus and AAV sequences. Once the virus or virus conjugate is taken up by a cell, the AAV ITR flanked transgene must be rescued from the parental adenovirus backbone. Rescue of the transgene is
15 dependent upon supplying the infected cell with an AAV rep gene.

The AAV rep gene can be supplied to the hybrid virus by several methods described in the above-incorporated application. One embodiment for
20 providing rep proteins in trans is by transfecting into the target monolayer of cells previously infected with the hybrid vector, a liposome enveloped plasmid containing the genes encoding the AAV rep 78 kDa and 52 kDa proteins under the control of the AAV P5 promoter.
25 More preferably for in vivo use, the AAV rep gene may also be delivered as part of the hybrid virus. One embodiment of this single particle concept is supplied by a polycation conjugate of hybrid virus. Infection of this modified virus conjugate is accomplished in the same
30 manner and with regard to the same target cells as identified above. However, the polylysine conjugate of the hybrid virus onto which was directly complexed a plasmid that encoded the rep 78 and 52 proteins, combines all of the functional components into a single particle
35 structure. Thus, the hybrid virus conjugate permits

delivery of a single particle to the cell, which is considerably more desirable for therapeutic use. In another embodiment, the hybrid virus is modified by cloning the rep cDNA directly into the adenovirus genome portion of the hybrid vector.

These and additional aspects of this hybrid vector are provided by the above-incorporated by reference application.

C. *Production of the Recombinant Viral*

10 *Particle*

1. Helper Viruses/Packaging Cell Lines

Depending upon the adenovirus gene content of the plasmid vectors employed to carry the VLDLR minigene, a packaging cell line or a helper adenovirus or both may be necessary to provide sufficient adenovirus gene sequences necessary to produce an infective recombinant viral particle containing the VLDLR minigene.

Useful helper viruses contain selected adenovirus gene sequences not present in the adenovirus vector construct or expressed by the cell line in which the vector is transfected. A preferred helper virus is desirably replication defective and contains a variety of adenovirus genes in addition to the modified sequences described above. In this setting, the helper virus is desirably used in combination with a packaging cell line that stably expresses adenovirus genes. Helper viruses may also be formed into poly-cation conjugates as described in Wu et al, J. Biol. Chem., 264:16985-16987 (1989); K. J. Fisher and J. M. Wilson, Biochem. J., 299:49 (April 1, 1994), and in U. S. Patent Application Serial No. 08/331,381, incorporated by reference herein.

Helper virus may optionally contain a second reporter minigene. A number of such reporter

genes are known to the art. The presence of a reporter gene on the helper virus which is different from the transgene on the adenovirus vector allows both the Ad vector and the helper virus to be independently
5 monitored. This second reporter is used to enable separation between the resulting recombinant virus and the helper virus upon purification. The construction of desirable helper cells is within the skill of the art.

As one example, if the cell line
10 employed to produce the viral vector is not a packaging cell line, and the vector contains only the minimum adenovirus sequences identified above, the helper virus may be a wild type Ad vector supplying the necessary adenovirus early genes E1, E2a, E4 and all remaining
15 late, intermediate, structural and non-structural genes of the adenovirus genome. However, if, in this situation, the packaging cell line is 293, which supplies the E1 proteins, the helper cell line need not contain the E1 gene.

20 In another embodiment, if the adenovirus vector construct is replication defective (no E1 gene and optionally no E3 gene) and the 293 cell line is employed, no helper virus is necessary for production of the hybrid virus. E3 may be eliminated from the
25 helper virus because this gene product is not necessary for the formation of a functioning virus particle.

Preferably, to facilitate purification and reduce contamination of the viral vector particle with the helper virus, it is useful to modify
30 the helper virus' native adenoviral gene sequences which direct efficient packaging, so as to substantially disable or "cripple" the packaging function of the helper virus or its ability to replicate.

A desirable "crippled" adenovirus is
35 modified in its 5' ITR packaging/enhancer domain, which

normally contains at least seven distinct yet functionally redundant sequences necessary for efficient packaging of replicated linear adenovirus genomes ("PAC" sequences). Within a stretch of nucleotide sequence from
5 bp 194-358 of the Ad5 genome, five of these PAC sequences are localized: PAC I or its complement at bp 241-248 [SEQ ID NO: 4], PAC II or its complement at bp 262-269 [SEQ ID NO: 5], PAC III or its complement at bp 304-311 [SEQ ID NO: 6], PAC IV or its complement at bp 314-321 [SEQ ID
10 NO: 7], and PAC V or its complement at bp 339-346 [SEQ ID NO: 8].

Mutations or deletions may be made to one or more of these PAC sequences in an adenovirus helper virus to generate desirable crippled helper
15 viruses. Modifications of this domain may include 5' adenovirus sequences which contain less than all five of the native adenovirus PAC sequences, including deletions of contiguous or non-contiguous PAC sequences. An alternative modification may be the replacement of one or
20 more of the native PAC sequences with one or more repeats of a consensus sequence containing the most frequently used nucleotides of the five native PAC sequences. Alternatively, this adenovirus region may be modified by deliberately inserted mutations which disrupt one or more
25 of the native PAC sequences. One of skill in the art may further manipulate the PAC sequences to similarly achieve the effect of reducing the helper virus packaging efficiency to a desired level.

It should be noted that one of skill
30 in the art may design other helper viruses or develop other packaging cell lines to complement the adenovirus deletions in the vector construct and enable production of the recombinant virus particle, given this information. Therefore, the use or description of any

particular helper virus or packaging cell line is not limiting.

In the presence of other packaging cell lines which are capable of supplying adenoviral proteins in addition to the E1, the helper virus may accordingly be deleted of the genes encoding these adenoviral proteins. Such additionally deleted helper viruses also desirably contain crippling modifications as described above.

Poly-cation helper virus conjugates, which may be associated with a plasmid containing other adenoviral genes, which are not present in the helper virus may also be useful. The helper viruses described above may be further modified by resort to adenovirus-polylysine conjugate technology. See, e.g., Wu et al, cited above; and K. J. Fisher and J. M. Wilson, cited above.

Using this technology, a helper virus containing preferably the late adenoviral genes is modified by the addition of a poly-cation sequence distributed around the capsid of the helper virus. Preferably, the poly-cation is poly-lysine, which attaches around the negatively-charged vector to form an external positive charge. A plasmid is then designed to express those adenoviral genes not present in the helper virus, e.g., the E1, E2 and/or E4 genes. The plasmid associates to the helper virus-conjugate through the charges on the poly-lysine sequence. This conjugate permits additional adenovirus genes to be removed from the helper virus and be present on a plasmid which does not become incorporated into the virus during production of the recombinant viral vector. Thus, the impact of contamination is considerably lessened.

2. Assembly of Viral Particle and Infection of a Cell Line

Assembly of the selected DNA sequences of the adenovirus, the AAV and the reporter genes or therapeutic genes and other vector elements into the hybrid vector and the use of the hybrid vector to produce a hybrid viral particle utilize conventional techniques. Such techniques include conventional cloning techniques of cDNA such as those described in texts [Sambrook et al, cited above], use of overlapping oligonucleotide sequences of the adenovirus and AAV genomes, polymerase chain reaction, and any suitable method which provides the desired nucleotide sequence. Standard transfection and co-transfection techniques are employed, e.g., CaPO₄ transfection techniques using the complementation 293 cell line. Other conventional methods employed include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

For example, following the construction and assembly of the desired minigene-containing plasmid vector, the vector is infected in vitro in the presence of an optional helper virus and/or a packaging cell line. Homologous recombination occurs between the helper and the vector, which permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the recombinant vector viral particles. The current method for producing such virus particles is transfection-based. Briefly, helper virus is used to infect cells, such as the packaging cell line human HEK 293, which are then subsequently transfected with an adenovirus plasmid vector containing a VLDLR transgene by conventional methods. About 30 or more hours post-transfection, the cells are harvested, an extract

prepared and the recombinant virus vector containing the *VLDLR* transgene is purified by buoyant density ultracentrifugation in a CsCl gradient.

5 The yield of transducing viral particles is largely dependent on the number of cells that are transfected with the plasmid, making it desirable to use a transfection protocol with high efficiency. One such method involves use of a poly-L-lysinylated helper adenovirus as described above. A
10 plasmid containing the *VLDLR* minigene is then complexed directly to the positively charged helper virus capsid, resulting in the formation of a single transfection particle containing the plasmid vector and the helper functions of the helper virus.

15 *II. Use of the Recombinant Virus Vectors in Gene Therapy*

The resulting recombinant adenoviral vector containing the *VLDLR* minigene produced by cooperation of the adenovirus vector and helper virus or adenoviral vector and packaging cell line, as described above, thus
20 provides an efficient gene transfer vehicle which can deliver the *VLDLR* gene to a patient *in vivo* or *ex vivo* and provide for integration of the gene into a liver cell.

The above-described recombinant vectors are
25 administered to humans in a conventional manner for gene therapy and serve as an alternative or supplemental gene therapy for LDL receptor deficiencies or other lipoprotein metabolic disorders. A viral vector bearing the *VLDLR* gene may be administered to a patient,
30 preferably suspended in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions

known to be pharmaceutically acceptable carriers and well known to those of skill in the art may be employed for this purpose.

The viral vectors are administered in
5 sufficient amounts to transfect the liver cells and provide sufficient levels of transfer and expression of the *VLDLR* gene to provide a therapeutic benefit without undue adverse or with medically acceptable physiological effects which can be determined by those skilled in the
10 medical arts. Conventional and pharmaceutically acceptable routes of administration include direct delivery to the liver, intranasal, intravenous, intramuscular, subcutaneous, intradermal, oral and other parental routes of administration. Routes of
15 administration may be combined, if desired.

Dosages of the viral vector will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. For example, a therapeutically
20 effective human dosage of the viral vector is generally in the range of from about 20 to about 100 ml of saline solution containing concentrations of from about 1×10^9 to 1×10^{11} pfu/ml virus vector. A preferred human dosage is estimated to be about 50 ml saline solution at 2×10^{10}
25 pfu/ml. The dosage will be adjusted to balance the therapeutic benefit against any adverse side effects. The levels of expression of the *VLDLR* gene can be monitored to determine the frequency of dosage administration.

30 An optional method step involves the co-administration to the patient, either concurrently with, or before or after administration of the viral vector, of a suitable amount of an immune modulator, which is preferably short-acting. The selected immune modulator
35 is defined herein as an agent capable of inhibiting the

formation of neutralizing antibodies directed against products of the recombinant vector of this invention and/or capable of inhibiting cytolytic T lymphocyte (CTL) elimination of the vector containing cells. The immune
5 modulator may interfere with the interactions between the T helper subsets (T_{H1} or T_{H2}) and B cells to inhibit neutralizing antibody formation. Alternatively, the immune modulator may be selected to inhibit the interaction between T_{H1} cells and CTLs to reduce the
10 occurrence of CTL elimination of the vector. More specifically, the immune modulator desirably interferes with, or blocks, the function of the CD4 T cells.

Immune modulators for use in inhibiting neutralizing antibody formation may be selected based on
15 the determination of the immunoglobulin subtype of any neutralizing antibody produced in response to the VLDLR-containing adenovirus vector. For example, if the neutralizing antibody is a T_{H2} mediated antibody, such as IgA, the immune modulator desirably suppresses or
20 prevents the interaction of T_{H2} with B cells.

Alternatively, if the induced neutralizing antibody is a T_{H1} mediated antibody, such as IgG_{2A}, the immune modulator desirably suppresses or prevents the interaction of T_{H1} with B cells.

25 The neutralizing antibody which develops in response to administration of a viral vector of this invention can be based on what vehicle is being used to deliver the vector and/or the location of delivery. For instance, administration of adenoviral vectors via the
30 lungs generally induces production of IgA neutralizing antibody. Administration of adenoviral vectors via the blood generally induces IgG, neutralizing antibody. The determination of the neutralizing antibody is readily determined in trials of the selected viral vector in
35 animal models. Where the reduction of CTL elimination of

the viral vectors is desired, the immune modulator is selected for its ability to suppress or block CD4⁺ T_{H1} cells to permit prolonged residence of the viral vector *in vitro*.

5 Selection of the immune modulator thus may be based upon the mechanism sought to be interrupted or blocked. The immune modulators may be soluble proteins or naturally occurring proteins, including cytokines, monoclonal antibodies. The immune modulators may be
10 conventional pharmaceuticals. The immune modulators identified herein may be used alone or in combination with one another. For example, cyclophosphamide and the more specific immune modulator anti-CD4 monoclonal antibody may be co-administered. In such a case,
15 cyclophosphamide serves as an agent to block T_{H1} activation and stabilized transgene expression beyond the period of transient immune blockade.

 A suitable amount or dosage of the immune modulator will depend primarily on the amount of the
20 recombinant vector bearing the VLDLR gene which is initially administered to the patient and the type of immune modulator selected. Other secondary factors such as the condition being treated, the age, weight, general health, and immune status of the patient, may also be
25 considered by a physician in determining the dosage of immune modulator to be delivered to the patient.

 Generally, for example, a therapeutically effective human dosage of a cytokine immune modulator, e.g., IL-12 or γ -IFN, is generally in the range of from
30 about 0.5 μ g to about 5 mg per about 1×10^7 pfu/ml virus vector. Various dosages may be determined by one of skill in the art to balance the therapeutic benefit against any side effects.

A. *Monoclonal Antibodies and Soluble Proteins*

Preferably, the method of inhibiting an adverse immune response to the gene therapy vector involves non-specific inactivation of CD4⁺ cells.

- 5 Preferably, such blocking antibodies are "humanized" to prevent the recipient from mounting an immune response to the blocking antibody. A "humanized antibody" refers to an antibody having its complementarily determining regions (CDRs) and/or other portions of its light and/or
10 heavy variable domain framework regions derived from a non-human donor immunoglobulin, the remaining immunoglobulin-derived parts of the molecule being derived from one or more human immunoglobulins. Such antibodies can also include antibodies characterized by a
15 humanized heavy chain associated with a donor or acceptor unmodified light chain or a chimeric light chain, or vice versa. Such "humanization" may be accomplished by methods known to the art. See, for example, G.E. Mark and E. A. Padlan, "Chap. 4. Humanization of Monoclonal
20 Antibodies", The Handbook of Experimental Pharmacology, vol. 113, Springer-Verlag, New York (1994), pp. 105-133, which is incorporated by reference herein.

- Other suitable antibodies include those that specifically inhibit or deplete CD4⁺ cells, such as
25 an antibody directed against cell surface CD4. Depletion of CD4⁺ cells has been shown by the inventors to inhibit the CTL elimination of the viral vector. Such modulatory agents include but are not limited to anti-T cell antibodies, such as anti-OKT3⁺ [see, e.g., US Patent No.
30 4,658,019; European Patent Application No. 501,233, published September 2, 1992]. See Example 2 below, which employs the commercially available antibody GK1.5 (ATCC Accession No. TIB207) to deplete CD4⁺ cells.

Alternatively, any agent that interferes with or blocks the interactions necessary for the activation of B cells by T_H cells, and thus the production of neutralizing antibodies, is useful as an immune modulator according to these methods. For example, B cell activation by T cells requires certain interactions to occur [F. H. Durie et al, Immunol. Today, 15(9):406-410 (1994)], such as the binding of CD40 ligand on the T helper cell to the CD40 antigen on the B cell, and the binding of the CD28 and/or CTLA4 ligands on the T cell to the B7 antigen on the B cell. Without both interactions, the B cell cannot be activated to induce production of the neutralizing antibody.

The CD40 ligand (CD40L)-CD40 interaction is a desirable point to block the immune response to gene therapy vectors because of its broad activity in both T helper cell activation and function as well as the absence of redundancy in its signaling pathway. A currently preferred method of the present invention thus involves transiently blocking the interaction of CD40L with CD40 at the time of adenoviral vector administration. This can be accomplished by treating with an agent which blocks the CD40 ligand on the T_H cell and interferes with the normal binding of CD40 ligand on the T helper cell with the CD40 antigen on the B cell. Blocking CD40L-CD40 interaction prevents the activation of the T helper cells that contributes to problems with transgene stability and readministration.

Thus, an antibody to CD40 ligand (anti-CD40L) [available from Bristol-Myers Squibb Co; see, e.g., European patent application 555,880, published August 18, 1993] or a soluble CD40 molecule can be a selected immune modulator in this method.

Alternatively, an agent which blocks the CD28 and/or CTLA4 ligands present on T helper cells interferes with the normal binding of those ligands with the antigen B7 on the B cell. Thus, a soluble form of B7 or an antibody to CD28 or CTLA4, e.g., CTLA4-Ig [available from Bristol-Myers Squibb Co; see, e.g., European patent application 606,217, published July 20, 1994] can be the selected immune modulator in the method of this invention. This method has greater advantages than the below-described cytokine administration to prevent T_{H2} activation, because it addresses both cellular and humoral immune responses to foreign antigens.

B. Cytokines

Still other immune modulators which inhibit the T_H cell function may be employed in this invention.

Thus, in one embodiment, an immune modulator which selectively inhibits the function of the T_{H1} subset of $CD4^+$ T helper cells may be administered at the time of primary administration of the viral vector. One such immune modulator is interleukin-4 (IL-4). IL-4 enhances antigen specific activity of T_{H2} cells at the expense of the T_{H1} cell function [see, e.g., Yokota et al, Proc. Natl. Acad. Sci., USA, 83:5894-5898 (1986); United States Patent No. 5,017,691]. It is envisioned that other immune modulators that can inhibit T_{H1} cell function will also be useful in the methods of this invention.

In another embodiment, the immune modulator can be a cytokine that prevents the activation of the T_{H2} subset of T helper cells. The success of this method depends on the relative contribution that T_{H2} dependent Ig isotypes play in virus neutralization, the profile of which may be affected by strain, the species

of animal as well as the mode of virus delivery and target organ.

A desirable immune modulator which selectively inhibits the CD4⁺ T cell subset T_{H2} function at the time of primary administration of the viral vector includes interleukin-12 (IL-12). IL-12 enhances antigen specific activity of T_{H1} cells at the expense of T_{H2} cell function [see, e.g., European Patent Application No. 441,900; P. Scott, Science, 260:496-497 (1993); R. Manetti et al, J. Exp. Med., 177:1199 (1993); A. D'Andrea et al, J. Exp. Med., 176:1387 (1992)]. IL-12 for use in this method is preferably in protein form. Human IL-12 may be recombinantly produced using known techniques or may be obtained commercially. Alternatively, it may be engineered into a viral vector (which optionally may be the same as that used to express the transgene) and expressed in a target cell in vivo or ex vivo.

T_{H2} specific ablation with IL-12 is particularly effective in lung-directed gene therapies where IgA is the primary source of neutralizing antibody. In liver-directed gene therapy, both T_{H1} and T_{H2} cells contribute to the production of virus specific antibodies. However, the total amount of neutralizing antibody can be diminished with IL-12.

Another selected immune modulator which performs a similar function is gamma interferon (IFN- γ) [S. C. Morris et al, J. Immunol., 152:1047-1056 (1994); F. P. Heinzel et al, J. Exp. Med., 177:1505 (1993)]. IFN- γ is believed to mediate many of the biological effects of IL-12 via secretion of activated macrophages and T helper cells. IFN- γ also partially inhibits IL-4 stimulated activation of T_{H2}. IFN- γ may also be obtained from a variety of commercial sources.

Alternatively, it may be engineered into a viral vector and expressed in a target cell *in vivo* or *ex vivo* using known genetic engineering techniques.

Preferably, such cytokine immune modulators are in the form of human recombinant proteins. These proteins may be produced by methods extant in the art. Active peptides, fragments, subunits or analogs of the known immune modulators described herein, such as IL-12 or gamma interferon, which share the T_H inhibitory function of these proteins, will also be useful in this method when the neutralizing antibodies are T_H mediated.

C. Other Pharmaceuticals

Other immune modulators or agents that non-specifically inhibit immune function, i.e., cyclosporin A or cyclophosphamide, may also be used in the methods of the invention. For example, a short course of cyclophosphamide has been demonstrated to successfully interrupt both CD4 and CD8 T helper cell activation to adenovirus capsid protein at the time of virus delivery to the liver. As a result, transgene expression was prolonged and, at higher doses, formation of neutralizing antibody was prevented, allowing successful vector readministration. In the lung, cyclophosphamide prevented formation of neutralizing antibodies at all doses and stabilized transgene expression at high dose.

D. Administration of Immune Modulator

The optional administration of the selected immune modulator may be repeated during the treatment with the recombinant adenovirus vector carrying the human *VLDLR* gene, during the period of time that the *VLDLR* gene is expressed (as monitored by e.g., *LDL* levels), or with every booster of the recombinant vector.

Thus, the compositions and methods of this invention provide a desirable treatment for defects in LDL metabolism, by providing stable expression of the VLDLR gene in human hepatocytes, and the ability to re-
5 administer the vector as desired without incurring an undesired immune response by the patient.

The following examples illustrate the construction and testing of the viral vectors and VLDL receptor gene inserts of the present invention and the
10 use thereof in the treatment of metabolic disorders. An exemplary recombinant adenovirus encoding the human VLDL receptor was constructed as described in Example 1 below. These examples are illustrative only, and do not limit the scope of the present invention.

15 Example 1 - Construction and Purification of H5.010CMVVLDLR

The cDNA for the human very low density lipoprotein (VLDL) receptor [M. E. Gafvels et al, cited above; SEQ ID NO: 1] was inserted into the polylinker
20 region of plasmid pRc/CMV (obtained from Invitrogen Corp.). The resulting plasmid, pRc/CMVVLDLR, was digested with the restriction enzymes SnaBI and NotI and the 4 kb fragment containing the cytomegalovirus (CMV) immediate-early promoter and VLDL receptor cDNA was
25 isolated.

The plasmid pAd.CMVlacZ [Kozarsky II, cited above] was digested with SnaBI and NotI to remove the CMV promoter and lacZ cDNA and the 5.6 kb backbone was isolated. The two fragments were ligated to generate
30 pAd.CMVVLDLR (Figs. 2 and 9; SEQ ID NO: 3). pAd.CMVVLDLR was linearized with NheI and co-transfected into 293 cells with sub360 DNA (derived from adenovirus type 5) which had been digested with XbaI and ClaI as previously described [K. F. Kozarsky I and II cited above].

The resulting recombinant adenovirus, designated H5.010CMVVLDLR, contains the sequence from about nucleotide 12 to about 4390 of pAd.CMVVLDLR and Ad.5 map units 9-100 with a small deletion in the E3 gene (see GenBank Accession No. M73260 and discussion of Fig. 3). This recombinant adenovirus was isolated following two rounds of plaque purification. H5.010CMVVLDLR was grown on 293 cells and purified by two rounds of cesium chloride density centrifugation as previously described [K. F. Kozarsky I and II cited above]. Cesium chloride was removed by passing the virus over a BioRad 10DG desalting column equilibrated with phosphate-buffered saline.

For rabbit experiments, virus was used freshly purified; for mouse experiments, virus was either used fresh, or after column purification glycerol was added to a final concentration of 10% (v/v), and virus was stored at -70°C until use.

As described in the following examples, this recombinant adenovirus vector was introduced into the livers of WHHL rabbits and into the livers of LDL receptor knockout mice to determine the *in vivo* function of the VLDL receptor, and to determine its usefulness as an alternative or supplemental gene therapy for LDL receptor deficiency.

Example 2 - Other Recombinant Adenoviruses

H5.010CMVlacZ, encoding the lacZ gene under the control of the CMV enhancer/promoter, and H5.010CBhLDLR, encoding the human low density lipoprotein (LDL) receptor cDNA under the control of the CMV-enhanced chicken β -actin promoter, were prepared as previously described [K. F. Kozarsky I and II, cited above].

Example 3 - Effects of Hepatic Expression of the VLDL Receptor in the WHHL Rabbit

H5.010CMVVLDLR or H5.010CMVlacZ (encoding β -galactosidase), obtained as described in Examples 1 and 2, was infused intravenously into WHHL rabbits [Camm Research] as follows. Rabbits were infused with 7.5×10^{12} particles of either recombinant adenovirus through a marginal ear vein on day 0. In addition, two New Zealand White (NZW) rabbits [Hazleton, Inc.] were infused with each virus and sacrificed on day 5 post-infusion to document the extent of gene transfer in the liver.

Rabbits were maintained in a 12 hour light/dark cycle on a diet of Purina laboratory chow, delivered each day at approximately 11:00 am. Venous samples were obtained through a marginal ear vein at approximately 10:00 am on the days indicated.

A. Plasma Analyses

Plasma samples were analyzed for total cholesterol using the Cholesterol HP kit and Precise standards (Boehringer Mannheim). Briefly, FPLC analysis was performed on 50 μ l of plasma from individual mice adjusted to a volume of 250 μ l in FPLC column buffer (1 mM EDTA, 154 mM NaCl, pH 8.0). Diluted samples (200 μ l) were loaded onto two Superose 6 columns (Pharmacia) in series at a flow rate of 0.4 ml/min, and 1 ml fractions were collected. Cholesterol content was analyzed in a microplate assay on 100 μ l samples. 100 μ l of a freshly prepared solution containing 50 mM PIPES, pH 6.9, 7.8 g/L HDCBS, 0.51 g/L 4-AAT, 1.27 g/L cholic acid, 0.245% Triton X-100, 7.31 g/L KCl and supplemented with 1.22 U/ml cholesterol oxidase, 7.64 U/ml cholesterol esterase, and 245 U/ml peroxidase was added to samples, incubated overnight at room temperature, and the O.D. at 490 nm was determined.

Plasma cholesterol levels were evaluated in each of the WHHL rabbits before and after receiving recombinant adenovirus. Fig. 4A shows that rabbits infused with H5.010CMVlacZ had no significant changes in cholesterol levels. However, following infusion with H5.010CMVVLDLR, cholesterol levels dropped, with maximum decreases that ranged from 140 to 420 mg/dl (Fig. 4B). This demonstrated that expression of the VLDL receptor results in decreased cholesterol levels in LDL receptor-deficient rabbits.

B. Histochemical Analysis

Portions of liver were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Some portions were fresh-frozen, sectioned, fixed in glutaraldehyde, stained with X-gal and lightly counterstained with hematoxylin. Some fresh-frozen sections were fixed in methanol, and then stained with either a polyclonal anti- β -galactosidase antibody (5 prime-3 prime), a polyclonal anti-human LDL receptor antibody, or with a polyclonal anti-VLDL receptor antibody, followed by a fluorescein isothiocyanate-conjugated anti-rabbit antibody (Jackson ImmunoResearch) as previously described [K. F. Kozarsky I and II cited above]. Oil Red O staining was performed on fresh-frozen sections fixed for 1 minute in 37% formaldehyde, then rinsed and stained in Oil Red O (3 parts 0.5% Oil Red O in isopropyl alcohol/2 parts water) for 10 minutes. Slides were counterstained in hematoxylin and mounted in aqueous solution.

Immunofluorescence analysis of the infused rabbits showed that approximately 50% of hepatocytes from the rabbit infused with H5.010CMVlacZ expressed β -galactosidase, liver tissue from the rabbit infused with H5.010CMVVLDLR had a slightly higher percentage of hepatocytes expressing the VLDL receptor. In agreement

with Northern blot analysis showing little or no VLDL receptor mRNA expression [M. E. Gafvels et al, cited above], liver from the lacZ-infused rabbit showed no reactivity with the anti-VLDL receptor antibody.

5 Example 4 - Effects of Short-Term Hepatic Expression of the VLDL Receptor in LDL Receptor Knockout Mice

C57BL/6 mice and LDL receptor knockout mice (Jackson Labs) were infused intravenously with 0.5 or 1.0 x 10¹⁰ particles of recombinant adenovirus through the
10 tail vein and cholesterol levels were monitored before and after infusion.

Specifically, three mice each were infused with either H5.010CMVlacZ, H5.010CMVVLDR, or H5.010CBhLDLR (encoding the human LDL receptor cDNA). This last virus
15 was included as a control to confirm published results [Kozarsky I and II cited above]. Plasma samples were obtained by retro-orbital bleeds using heparinized capillary tubes. The LDL receptor knockout mice were maintained upon a high cholesterol diet composed of
20 Purina mouse chow supplemented with 1.25% cholesterol, 7.5% cocoa butter, 7.5% casein, and 0.5% cholate (1.25% cholesterol diet) for at least 3 weeks immediately following weaning before experiments were initiated. Mice were sacrificed on day 5 post-infusion.

25 Liver tissues were analyzed by immunofluorescence for transgene expression by the techniques described in Example 3, and plasma cholesterol levels were measured as similarly described. For lipoprotein fractionations, plasma from triplicate LDL
30 receptor knockout mice were pooled, subjected to density ultracentrifugation, fractions were collected, and the cholesterol content was determined by conventional means.

Immunofluorescence analysis revealed moderate levels of β -galactosidase expression in H5.010CMVlacZ-infused mice, and higher levels of either human LDL receptor and VLDL receptor expression in H5.010CBhLDLR-
5 and in H5.010CMVVLDLR-infused mice, respectively.

Cholesterol levels decreased slightly in the control, H5.010CMVlacZ-infused mice (Fig. 5), probably due to non-transgene-related effects of infusion of recombinant adenovirus, which can result in
10 hepatotoxicity in mice [Y. Yang et al, Proc. Natl. Acad. Sci., USA, 91:4407-4411 (May 1994)]. However, in contrast to the decrease observed in the control mice, cholesterol levels dropped significantly to 50% of pre-infusion values in the H5.010CBhLDLR-infused mice on day
15 5 post-infusion. Cholesterol levels in the H5.010CMVVLDLR-infused mice also decreased, to approximately 60% of pre-infusion levels. Further analysis of plasma lipoproteins showed that in the H5.010CBhLDLR-treated mice, LDL levels plummeted, with
20 additional decreases in IDL and VLDL fractions (Fig. 6). The H5.010CMVVLDLR-infused mice showed a larger decrease in the VLDL fraction with less of a decrease in LDL.

Taken together, these data indicate that hepatic expression of VLDL receptor results in increased
25 clearance of VLDL from the plasma, resulting in decreases in the amounts of lipoproteins for which VLDL is the precursor (i.e., IDL and LDL), and an overall drop in total plasma cholesterol.

30 Example 5 - Effects of Long-Term Hepatic Expression of the VLDL Receptor in LDL Receptor Knockout Mice

In order to achieve cholesterol levels closer to those observed in both FH patients and WHHL rabbits, LDL receptor knockout mice (Jackson Labs) were maintained on a high cholesterol diet composed of Purina mouse chow

supplemented with 0.2% cholesterol, 10% coconut oil, and 0.05% cholate (0.2% cholesterol diet). Cholesterol levels in these mice ranged from 930 to 1550 mg/dl, whereas the mice on the 1.25% cholesterol (Example 4) diet had levels of 1900 to 3100 mg/dl.

Virus was thawed immediately before use and diluted with PBS to a concentration of 1×10^{12} particles/ml. Three mice were each infused intravenously with 0.1 ml of virus containing 1×10^{11} particles of an E1-deleted recombinant adenovirus encoding either β -galactosidase (H5.010CMVlacZ) or human LDL receptor (H5.010CBhLDLR), and serum lipids were followed over time. On the days indicated, mice were anesthetized with methoxyflurane and blood was collected into heparinized capillary tubes by puncture of the retro-orbital venous plexus.

Immunofluorescence staining showed that most of the hepatocytes expressed the transgene product, either β -galactosidase, human LDL receptor, or VLDL receptor. Hematoxylin and eosin staining of sections of liver revealed essentially normal morphology in the H5.010CMVlacZ-infused mouse. However, for both the H5.010CBhLDLR- and H5.010CMVVLDLR-infused mice, hepatocytes appeared to have internal vacuoles. When tissue was analyzed with Oil Red O staining, a stain for neutral lipids, liver from the receptor-infused animals clearly showed accumulation of large droplets of lipid when compared with the H5.010CMVlacZ-infused control. This suggested that short-term, high level expression of the LDL receptor or VLDL receptor in these LDL receptor-deficient mice resulted in intracellular accumulation of lipids.

To confirm the biological activities of the transgene products, plasma cholesterol levels were followed before and after recombinant adenovirus

administration. Fig. 7A shows that serum cholesterol levels in H5.010CMVlacZ-infused mice demonstrated a characteristic but not significant fluctuation over time, reflected in minor changes of all lipoprotein fractions (HDL, IDL/VLDL, and LDL). In contrast, mice infused with H5.010CBhLDLR have a large but transient decrease in cholesterol (see, Fig. 7B). Particularly, these mice demonstrated large plasma cholesterol decreases which lasted for approximately 2 weeks. Cholesterol levels decreased 3-fold (from 966 to 353 mg/dl) and 7-fold (from 1554 to 219 mg/dl) and returned to baseline by 3 weeks post-infusion. The decrease in serum cholesterol is reflected in coordinate diminution in serum LDL. This nonspecific effect of the adenovirus infection when immune modulators are not coordinately administered has been described previously and is likely due to changes in hepatic function that occur as a result of the associated inflammation. Mice infused with H5.010CMVVLDLR showed large decreases in plasma cholesterol which were similar in magnitude to those seen in the H5.010CBhLDLR-infused mice (Fig. 7C), with maximum decreases of more than 4-fold (from 1186 to 288 mg/dl and from 1453 to 299 mg/dl). Surprisingly, plasma cholesterol levels did not return to baseline by 3 weeks post-infusion. The change in plasma cholesterol levels in the H5.010CMVVLDLR-infused mice (Fig. 7A) were statistically significant ($p < 0.05$) through 9 weeks following infusion (the current duration of the experiment).

Sera from individual mice was analyzed by FPLC to determine the effects of VLDL receptor expression on lipoprotein fractions. On day 3 post-infusion, VLDL and LDL fractions were undetectable; over time, the LDL fraction slowly recovered, although even at 10 weeks post-infusion, the LDL peak height was slightly lower than the HDL peak height. VLDL remained undetectable

although minor differences may escape detection because of limitations in the sensitivity of the cholesterol assay. The LDL peaks mirrored the total plasma cholesterol levels, and confirmed that the prolonged
5 lowering of plasma cholesterol was accompanied by sustained decreases in VLDL and LDL levels. These data suggest that expression of the VLDL receptor in the liver is an effective therapy for hypercholesterolemia.

At the same time of infusion of the LDL
10 receptor knockout mice, normal C57Bl/6 mice were infused with each of the recombinant adenoviruses. These mice were sacrificed at various times post-infusion, and liver tissues were harvested for direct analysis of transgene expression using X-gal histochemistry to detect β -
15 galactosidase expression and immunofluorescence performed to measure LDL receptor expression. Tissues harvested three days after infusion of virus demonstrated either expression of β -galactosidase or the human LDL receptor in at least 80% of hepatocytes.

20 In each experiment, the vector specific signal was substantially higher than that seen in animals before gene transfer or following infusion with identical quantities of an adenovirus expressing an irrelevant gene. For both lacZ and LDL receptor, transgene
25 expression diminished to undetectable levels by day 21 and was associated with the development of a self limited mononuclear infiltrate in liver that peaked at day 10. The infiltrate consisted of portal as well as lobular inflammation, accompanied by the presence of apoptic
30 bodies. The extent of pathology was indistinguishable between the lacZ and LDL receptor infused mice. The time course of LDL receptor expression is consistent with the initial large decline in plasma cholesterol and subsequent return to baseline.

In contrast, two mice infused with H5.010CMVVLDLR expressed the VLDL receptor at high levels. The percent of hepatocytes may have decreased slightly as compared to the day 5 mice. These data suggest that the sustained decrease in plasma cholesterol levels in the H5.010CMVVLDLR-infused mice was due to sustained expression of the VLDL receptor.

Example 6 - Turnover Studies

To further characterize the effects of hepatic VLDL receptor expression on lipoprotein metabolism, turnover studies were performed as follows.

LDL receptor knockout mice were infused with recombinant adenovirus after 3 weeks on the high cholesterol diet as described in Example 4. Three mice each were injected with the lacZ and VLDL receptor adenoviruses; one mouse was injected with the LDL receptor adenovirus. On day 5 post-infusion, mice were injected via the tail vein with approximately 8×10^6 cpm of ^{125}I -labeled human LDL, and 1.6×10^5 cpm of ^{131}I -labeled human VLDL in a total volume of 0.2 ml. A blood sample was obtained 1 minute following injection of radiolabel, and designated the "time zero" sample. Blood was collected into heparinized capillary tubes at the indicated times, and radioactivity remaining was determined using a gamma counter.

Infusion of LDL receptor adenovirus led to accelerated clearance of LDL as compared to infusion of lacZ adenovirus, consistent with a previous study in LDL receptor knockout mice [S. Ishibashi et al, J. Clin. Invest., 92:883-893(1993)]. Similarly, VLDL clearance was accelerated in LDL receptor treated animals as compared to lacZ infused mice. LDL turnover in VLDL receptor-infused mice was indistinguishable from lacZ infused mice, consistent with *in vitro* data which

indicates that LDL is not a ligand for the VLDL receptor [T. Yamamoto et al, Trends in Cardiovascular Medicine, 3:144-148 (1993); F. Batley et al, J. Biol. Chem., 269:23268-23273 (1994)]. VLDL clearance in VLDL receptor
5 infused mice was slightly faster than in lacZ infused mice, but significantly slower than in LDL receptor infused mice.

As discussed above, VLDL turnover in mice infused with the VLDL receptor adenovirus was
10 significantly faster than in lacZ infused mice although the magnitude of this effect was far less than that seen in animals treated with LDL receptor virus. This suggests that VLDL receptor-mediated clearance of circulating VLDL may not be the only pathway leading to
15 diminished serum VLDL. One potential mechanism is secretion-recapture, which occurs with hepatic uptake of chylomicron remnants [T. Willnow & J. Herz, J. Mol. Med., 73:213-220 (1995); H. Shimano et al, J. Clin. Invest., 93:2215-2223 (1994)], and would result in decreased
20 secretion of VLDL and reduced levels of plasma VLDL. A second mechanism may involve the interaction of the VLDL receptor with receptor-associated protein (RAP) [Batley, cited above; H. Mokuno et al, J. Biol. Chem., 269:13238-13243 (1994)] which interacts with a variety of receptors
25 inside the cell, apparently to prevent ligand binding before the receptor reaches the cell surface [G. Bu et al, EMBO J, 14:2269-2280 (1995)]. It is possible that the high levels of VLDL receptor expressed in the livers of adenovirus-infused mice overwhelms the available RAP,
30 so that VLDL receptor is binding to newly synthesized ligand (apoE, either free or in association with lipid) within the cell, and preventing its secretion into the plasma. The effects of hepatic VLDL receptor expression on total plasma cholesterol as well as on lipoprotein

cholesterol levels demonstrate that the VLDL receptor can play a major role in lipoprotein metabolism in vivo.

Example 7 - Stability of Expression of VLDL Receptor

5 This experiment illustrates relative transgene persistence in mice.

LDL receptor knockout mice were injected intravenously on day 0 with 1×10^{11} particles of H5.010CMVlacZ, H5.010CBhLDLR, or H5.010CMVVLDLR. Mice were sacrificed on the indicated days after injection (3, 10 or 21), and fresh-frozen sections of liver were stained with X-gal to detect expression of the lacZ gene, and with anti-LDL receptor antibody or anti-VLDL receptor antibody, followed by a fluorescein-conjugated secondary antibody to detect LDL receptor and VLDL receptor, respectively.

Analysis of liver harvested 3 days after infusion of virus revealed VLDL receptor protein in >80% of hepatocytes; the bright fluorescent signal, which localized to the perimeter of the cell, was absent before gene transfer and in tissues of animals infected with lacZ or LDL receptor containing adenoviruses. Expression of VLDL receptor protein was remarkably stable with recombinant protein detected in approximately 5 to 10% of hepatocytes from tissue harvested 105 days after infusion of virus. This is in striking contrast to the results obtained with lacZ and LDL receptor adenovirus, where expression of the transgene extinguished to undetectable levels within three weeks of gene transfer. VLDL receptor expression remained detectable through the duration of the experiment (22 weeks).

Genomic DNA was isolated from mouse liver, digested with EcoRI, and subjected to Southern blotting [K. Kozarsky et al. J. Biol. Chem., 269:13695-13702 (1994)] to monitor the presence over time of adenoviral

DNA sequences. Adenovirus sequences were detected using the Genius kit from Boehringer Mannheim, followed by chemiluminescent detection. In C57BL/6 mice infused with the lacZ adenovirus, viral DNA diminished rapidly with time, plateauing at barely detectable levels (~ 0.05 copies/cell) through day 70 post-infusion. Mice infused with VLDL receptor had slightly higher initial levels of DNA, but a similar time course of loss of adenovirus sequences. Additional DNA hybridization studies showed that the majority of adenovirus DNA initially delivered to the liver is not integrated into the mouse genome (data not shown), however, this assay cannot rule out some level of integration.

Histopathologic analysis of liver tissue from mice infused with the VLDL receptor virus revealed inflammation and apoptotic cells at early time points. The timing and extent of the pathologic findings were indistinguishable from liver tissues of mice infused with lacZ and LDL receptor viruses. At 15 and 22 weeks post-infusion, however, liver tissue from VLDL receptor-infused mice displayed discernible accumulations of neutral lipids, as demonstrated by hematoxylin and eosin as well as oil red O staining. Similar changes were observed infrequently in LDL receptor knockout mice infused with PBS, LDL receptor and/or lacZ adenoviruses. No lipid accumulations were observed in livers of normal C57BL/6 mice infused with the VLDL receptor virus, despite long-term transgene expression indistinguishable from that observed in LDL receptor knockout mice. This indicates that VLDL receptor expression alone is not sufficient for the changes in lipid accumulation observed in LDL receptor knockout mice; instead, there is some lipid accumulation in the LDL receptor knockout mice which have been maintained on a high cholesterol diet for

≥ 18 weeks, that is accelerated by prolonged VLDL receptor expression.

Plasma samples from mice infused with VLDL receptor adenovirus were analyzed for the presence of antibodies directed against the VLDL receptor protein. Only one mouse out of twelve generated antibodies to the VLDL receptor despite the presence of high level antibodies to adenovirus capsid proteins in each animal that received virus. Animals infused with the VLDL receptor adenovirus mounted a CTL response to adenoviral proteins indistinguishable from that obtained from animals infused with either lacZ or LDL receptor adenoviruses. These mice, however, did not mount a CTL response to the VLDL receptor protein. Thus, the development of a CTL response to the transgene following infusion of recombinant adenovirus is dependent on the antigenicity of the specific transgene in the treated animal.

Example 8 - Humoral and Cellular Immune Response to Adenovirus and Transgenes

A. Humoral Immune Response

Two LDL receptor knockout mice (K020 and K027) or two normal C57BL/6 mice were injected via the tail vein with 1×10^{11} particles of H5.010CBhLDLR at day 0 and serum samples were collected both before injection (pre), and on days 10, 24, 39, 52 and 70 following injection for the knockout mice and on day 21 for the C57BL/6 mice. Western blots were performed as previously described [K. Kozarsky et al, J. Biol. Chem., 269:13695-13702 (1994); K. Kozarsky et al., Som. Cell and Molec. Genet., 19:449-458 (1993)]. To detect anti-adenovirus antibodies, purified adenovirus was used as the antigen.

The positive control (+) was rabbit antiserum isolated following intravenous infusion of purified H5.010CBhLDLR. The negative control (-) was pre-immune rabbit serum. Western blots with β -galactosidase were performed using purified protein (Sigma), with a monoclonal antibody specific for β -galactosidase (Sigma) as a positive control.

Antibodies directed against the human LDL receptor were detected using lysates prepared from 24-23 cells, a 3T3 cell line which was transduced with retrovirus encoding the human LDL receptor. For detection of anti-VLDL receptor antibodies, a lysate was prepared from HeLa cells two days following infection with H5.010 CMVVLDLR.

All mice infused with 1×10^{11} particles of recombinant adenovirus developed antibodies to adenovirus capsid proteins, with major bands corresponding to hexon, penton and fiber. All mice infused with H5.010CBhLDLR developed antibodies to the human LDL receptor protein with LDL receptor knockout mice consistently developing higher titer antibodies than C57BL/6 mice. Antibodies from LDL receptor knockout mice cross-reacted with mouse LDL receptor protein, whereas antibodies from C57BL/6 mice (which express normal mouse LDL receptor) did not.

This suggests that the VLDL receptor, although the human and not the mouse sequence was used, was not immunogenic in these mice. The amino acid sequences of the human and mouse LDL receptors are approximately 78% identical, while the human and mouse VLDL receptors are >94% identical. This increased sequence similarity is likely to account for the absence of antibody development to the human VLDL receptor despite high level expression in the mouse liver as a result of infusion of H5.010CMVVLDLR.

These data demonstrate that animals can generate a humoral immune response specific for the transgene product as well as to the viral proteins encoded on the injected adenovirus. It also provides indirect evidence of antigen specific activation of T helper cells, which is normally required for development of mature, antibody-secreting B cells.

B. Cellular Immune Responses

This study analyzed animals following infusion with the LDL receptor adenovirus for activation of CTLs to both viral antigens and the transgene product, human LDL receptor.

CTL assays were performed as described in Y. Yang et al, Immunity, 1:433-442 (1994). Target cells expressing recombinant vaccinia proteins were generated by infecting with recombinant vaccinia were generated as follows. The VLDV receptor CDNA (in the pRC/CMV plasmid) was subcloned into the HindIII site of Bluescript KS+. The CFTR cDNA [J.R. Riordan et al, Science, 245:1066-1073 (1989)] was cloned into the PstI site of Bluescript KS+ (Stratagene). The LDL receptor cDNA in the pUC19 vector [T. Yamamoto et al, Cell, 39:27-38 (1984)] was excised with the restriction enzymes HindIII and Sac I and ligated into the HindIII and SacI sites of Bluescript KS+. Each of the cDNAs was then excised using the enzymes SacII and KpnI and cloned into the SacII and KpnI sites of a modified form of the vaccinia expression vector pSC11 [S. Chakrabarti et al, Molec. Cell. Biol., 5:3403-3409 (1985)]. The control recombinant vaccinia, VRG, expresses a rabies virus glycoprotein and was prepared as described in T. Wiktor et al, Proc. Natl. Acad. Sci. USA, 81: 7194-7198 (1984).

CTLs to specific targets were detected in a standard ⁵¹chromium (⁵¹Cr) release assay in which MHC compatible target cells were infected with either

recombinant adenovirus or vaccinia viruses that express single relevant gene products. Figure 10 presents both an example of a ⁵¹Cr release assay in which % specific lysis is measured as a function of increasing the effector to target ratio (Fig. 10B), as well as a summary of the cumulative data (Fig. 10A). Splenocytes from C57BL/6 mice infused with recombinant adenovirus containing either human LDL receptor or human CFTR were evaluated for their ability to lyse targets infected with either recombinant adenovirus, to measure activity to viral proteins, or with vaccinia virus containing LDL receptor, to measure activity to LDL receptor protein. Cytolytic activity was demonstrated with lymphocytes from animals infected with the LDL receptor virus to target cells infected with the same virus. No cytolysis was detected to mock infected targets supporting the specificity of the assay. These same effector cells demonstrated significant cytolytic activity to targets infected with LDL receptor vaccinia virus that was not present when infected with a control vaccinia. These experiments provide strong evidence for the presence of activated CTL to human LDL receptor in C57BL/6 mice following gene therapy.

Example 9 - Enhancement of Adenovirus Mediated Gene Transfer upon Second Administration by IL-12 and IFN- γ in Mouse Lung.

The recombinant adenoviruses H5.010CMVlacZ and H5.010CBALP (alkaline phosphatase gene expressed from the CMV enhanced β -actin promoter in the sub360 backbone) were used in this example. Each similar virus expresses a different reporter gene whose expression can be discriminated from that of the first reporter gene.

Female C57Bl/6 mice (6-8 week old) were infected with suspensions of H5.010CBALP (1×10^9 pfu in 50 μ l of PBS) via the trachea at day 0 and similarly with H5.010CMVlacZ at day 28. One group of such mice was used as a control. Another group of mice were acutely depleted of CD4⁺ cells by i.p. injection of antibody to CD4⁺ cells (GK1.5; ATCC No. TIB207, 1:10 dilution of ascites) at the time of the initial gene therapy (days - 3, 0, and +3). A third group of mice were injected with IL-12 (1 μ g intratracheal or 2 μ g, i.p. injections) at the time of the first administration of virus (days 0 and +1). A fourth group of mice were injected with gamma interferon (1 μ g intratracheal or 2 μ g, i.p. injections) at the time of the first administration of virus (days 0 and +1).

When mice were subsequently euthanized and necropsied at days 3, 28, or 31, lung tissues were prepared for cryosections, while bronchial alveolar lavage (BAL) and mediastinal lymph nodes (MLN) were harvested for immunological assays.

A. Cryosections

The lung tissues were evaluated for alkaline phosphatase expression by histochemical staining following the procedures of Y. Yang et al, cited above.

Instillation of alkaline phosphatase virus (10^9 pfu) into the airway of all groups of the C57Bl/6 mice resulted in high level transgene expression in the majority of conducting airways that diminishes to undetectable levels by day 28. Loss of transgene expression was shown to be due to CTL mediated elimination of the genetically modified hepatocytes [Y. Yang et al, cited above].

In the control mice, no recombinant gene expression was detected three days after the second administration of virus, i.e., day 31.

Administration of virus to the CD4⁺ depleted animals was associated with high level recombinant transgene expression that was stable for a month. Expression of the second virus was detectable on day 31.

Initial high level gene transfer diminished after about one month in the IL-12 treated mice; however, in contrast to the control, high level gene transfer to airway epithelial cells was achieved when virus was readministered to IL-12 treated animals at day 28, as seen in the day 31 results.

The gamma-interferon treated animals were virtually indistinguishable from the animals treated with IL-12 in that efficient gene transfer was accomplished upon a second administration of virus.

B. Immunological Assays - MLN

Lymphocytes from MLN of the control group and IL-12 treated group of C57Bl/6 mice harvested 28 days after administration of H5.010CBALP were restimulated in vitro with UV-inactivated H5.010CMVlacZ at 10 particles/cell for 24 hours. Cell-free supernatants were assayed for the presence of IL-2 or IL-4 on HT-2 cells (an IL-2 or IL-4-dependent cell line) [Y. Yang et al, cited above]. Presence of IFN- γ in the same lymphocyte culture supernatant was measured on L929 cells as described [Y. Yang et al, cited above]. Stimulation index (S.I.) was calculated by dividing ³H-thymidine cpm incorporated into HT-2 cells cultured in supernatants of lymphocytes restimulated with virus by those incorporated into HT-2 cells cultured in supernatants of lymphocytes incubated in antigen-free medium.

The results are shown in Table 1 below.

Table 1

5	<u>³H-Thymidine Incorporation (cpm±SD)</u>			IFN-γ liter (IU/ml) ^d	
	Medium	H5.010CMVlacZ	S.I.		
	C57Bl/6	175 ± 40	2084 ± 66	11.91	80
	anti-IL2 (1:5000)		523 ± 81	2.98	
10	anti-IL4 (1:5000)		1545 ± 33	8.83	
	C57Bl/6 +IL12	247 ± 34	5203 ± 28	21.07	160
15	anti-IL2 (1:5000)		776 ± 50	3.14	
	anti-IL4 (1:5000)		4608 ± 52	18.66	

20 Stimulation of lymphocytes from regional lymph nodes with both recombinant adenoviruses led to secretion of cytokines specific for the activation of both T_{H1} (i.e., IL-2 and IFN- γ) and T_{H2} (i.e., IL-4) subsets of T helper cells (Table 1).

25 Analysis of lymphocytes from the IL-12 treated animals stimulated *in vitro* with virus revealed an increased secretion of IL-2 and IFN- γ and a relative decreased production of IL-4 as compared to animals that did not receive IL-12 (i.e., ratio of IL-2/IL-4 was increased from 3 to 6 when IL-12 was used; Table 1).

30 C. Immunological Assays - BAL

BAL samples obtained from animals 28 days after primary exposure to recombinant virus were evaluated for neutralizing antibodies to adenovirus and anti-adenovirus antibody isotypes as follows. The same
35 four groups of C57Bl/6 mice, i.e., control, CD4⁺

depleted, IL-12 treated and IFN- γ treated, were infected with H5.010CBALP. Neutralizing antibody was measured in serially diluted BAL samples (100 μ l) which were mixed with H5.010CMVlacZ (1×10^6 pfu in 20 μ l), incubated for 1 hour at 37°C, and applied to 80% confluent Hela cells in 96 well plates (2×10^4 cells per well). After 60 minutes of incubation at 37°C, 100 μ l of DMEM containing 20% FBS was added to each well. Cells were fixed and stained for β -galactosidase expression the following day.

10 All cells were lacZ positive in the absence of anti-adenoviral antibodies.

Adenovirus-specific antibody isotype was determined in BAL by using enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 100 μ l of PBS containing 5×10^9 particles of H5.010CMVlacZ for 18 hours at 4°C. The wells were washed 5 times with PBS. After blocking with 200 μ l of 2% BSA in PBS, the plates were rinsed once with PBS and incubated with 1:10 diluted BAL samples for 90 minutes at 4°C. Thereafter, the wells were extensively washed and refilled with 100 μ l of 1:1000 diluted alkaline phosphatase-conjugated anti-mouse IgG or IgA (Sigma). The plates were incubated, subsequently washed 5 times, and 100 μ l of the substrate solution (p-nitrophenyl phosphate, PNPP) was added to each well. Substrate conversion was stopped by the addition of 50 μ l of 0.1M EDTA. Plates were read at 405 nm.

The results are shown graphically in Figs. 11A through 11C, which summarize neutralizing antibody titer, and the relative amounts (OD_{405}) of IgG and IgA present in BAL samples. The titer of neutralizing antibody for each sample was reported as the highest dilution with which less than 50% of cells stained blue.

As demonstrated in the first bar of Figs. 11A through 11C, the cytokines identified in Table 1 above were associated in the control mice with the appearance of antibodies to adenovirus proteins in BAL of both the IgG and IgA isotypes that were capable of neutralizing the human Ad5 recombinant vector in an *in vitro* assay out to a 1:800 dilution.

As shown in the second bar of the graphs of Figs. 11A through 11C, transient CD4⁺ cell depletion inhibited the formation of neutralizing antibody (Fig. 11A) and virus specific IgA antibody (Fig. 11C) by 80-fold, thereby allowing efficient gene transfer to occur following a second administration of virus. Fig. 11B shows a slight inhibition of IgG as well.

More importantly, as shown in the third bar of the three graphs, IL-12 selectively blocked secretion of antigen specific IgA (Fig. 11C), without significantly impacting on formation of IgG (Fig. 11B). This was concurrent with a 32-fold reduction in neutralizing antibody (Fig. 11A).

The gamma-interferon treated animals (fourth bar of Figs. 11A through 11B) were virtually indistinguishable from the animals treated with IL-12 in that virus specific IgA (Fig. 11C) and neutralizing antibody (Fig. 11A) were decreased as compared to the control animals not treated with cytokine, but not to the extent obtained with those treated with IL-12.

These studies demonstrate that inhibition of CD4⁺ function at the time of primary exposure to virus is sufficient to prevent the formation of blocking antibodies. The concordant reduction of neutralizing antibody with antiviral IgA suggests that immunoglobulin of the IgA subtype is primarily responsible for the blockade to gene transfer.

All references recited above are incorporated herein by reference. Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be
5 obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention, such as selections of different modifications of adenovirus vectors selected to carry the VLDLR gene, or selection or dosage of the vectors or
10 immune modulators are believed to be within the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Trustees of University of Pennsylvania
Wilson, James M.
Kozarsky, Karen F.
Strauss, Jerome F.
- (ii) TITLE OF INVENTION: Methods and Compositions for Gene
Therapy for the Treatment of Defects in Lipoprotein
Metabolism
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Howson and Howson
 - (B) STREET: Spring House Corporate Cntr., PO Box 457
 - (C) CITY: Spring House
 - (D) STATE: Pennsylvania
 - (E) COUNTRY: USA
 - (F) ZIP: 19477
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/393,734
 - (B) FILING DATE: 24-FEB-1995
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Bak, Mary E.
 - (B) REGISTRATION NUMBER: 31,215
 - (C) REFERENCE/DOCKET NUMBER: GNVPN009CIP1.PCT
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 215-540-9200
 - (B) TELEFAX: 215-540-5818

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3656 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: DNA (genomic)

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(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 392..3010

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCTGCGGGC CCGCGGTGCG GGTGCTGCT ACCGCTGTC TCGTTCTGT CCTCTCTTCT	60
CCTCTGCTGT CCCCACCGCC TGTCCGTTCC GTGCTGTCCC GTTCCGTCCC GTGCTGTCCA	120
CGCGCTGCA TATTTTCTGC CCGCAGCTCG GCTTCCACTG CTGCTGCAGC CCGCGGAGCT	180
CGCTCGCTCG GTGCGGAGCA GAGTGTCCAA GTTGTAGCGG ACCGCGTCCC GTCTTCTTCC	240
CGGCTGCGCT CCCCAGCCAA GTGCTTCCCG TCGTTGTCCC CTTTCCCGCT CCGACCGCCC	300
ACCTTCTTCC TCTTTCCGAA CCGCTGCTAA GTTGTGCTCC GAGCGGAGC CCGCGCGCGC	360
CGCGCGCGCG CCGACCATCC ACCCGCGGAC C ATG GCG ACC TCC GCG CTC TCG	412
Met Gly Thr Ser Ala Leu Trp	
1 5	
GCC CTC TCG CTG CTG CTC GCG CTG TCG TCG GCG CCC GCG GAG AGC GCG	460
Ala Val Trp Leu Leu Leu Ala Leu Cys Trp Ala Pro Arg Glu Ser Gly	
10 15 20	
GCC ACC GGA ACC GCG AGA AAA GCG AAA TGT GAA CCC TCC CAA TTC CAG	508
Ala Thr Gly Thr Gly Arg Lys Ala Lys Cys Glu Pro Ser Gln Phe Gln	
25 30 35	
TGC ACA AAT GGT GCG TGT ATT ACC CTG TTG TCG AAA TGT GAT GCG GAT	556
Cys Thr Asn Gly Arg Cys Ile Thr Leu Leu Trp Lys Cys Asp Gly Asp	
40 45 50 55	
GAA GAC TGT GTT GAC GCG AGT GAT GAA AAG AAC TGT GTA AAG AAG ACC	604
Glu Asp Cys Val Asp Gly Ser Asp Glu Lys Asn Cys Val Lys Lys Thr	
60 65 70	
TGT GCT GAA TCT GAC TTC CTG TCG AAC AAT GCG CAG TGT GTT CCC AGC	652
Cys Ala Glu Ser Asp Phe Val Cys Asn Asn Gly Gln Cys Val Pro Ser	
75 80 85	
GGA TCG AAG TGT GAT GGA GAT GGT GAC TCG GAA GAT GGT TCA GAT GAA	700
Arg Trp Lys Cys Asp Gly Asp Pro Asp Cys Glu Asp Gly Ser Asp Glu	
90 95 100	
AGC CCA GAA CAG TCG CAT ATG AGA ACA TCG GCG ATA CAT GAA ATC AGC	748
Ser Pro Glu Gln Cys His Met Arg Thr Cys Arg Ile His Glu Ile Ser	
105 110 115	
TGT GCG GCG CAT TCT ACT CAG TGT ATC CCA GTG TCC TCG AGA TGT GAT	796
Cys Gly Ala His Ser Thr Gln Cys Ile Pro Val Ser Trp Arg Cys Asp	
120 125 130 135	
GCT GAA AAT GAT TGT GAC AGT GGA GAA GAT GAA GAA AAC TGT GCG AAT	844
Gly Glu Asn Asp Cys Asp Ser Gly Glu Asp Glu Glu Asn Cys Gly Asn	
140 145 150	
ATA ACA TGT AGT CCC GAC GAG TTC ACC TCG TCC AGT GCG GCG TCG ATC	892
Ile Thr Cys Ser Pro Asp Glu Phe Thr Cys Ser Ser Gly Arg Cys Ile	
155 160 165	

TCC	AGG	AAC	TTT	GTA	TGC	AAT	CCC	CAG	GAT	GAC	TGC	AGC	GAT	CCC	AGT	940
Sor	Arg	Asn	Phe	Val	Cys	Asn	Gly	Gln	Asp	Asp	Cys	Sor	Asp	Gly	Sor	
	170						175					180				
GAT	GAG	CTG	GAC	TGT	CCC	CCC	CCA	ACC	TGT	CCC	CCC	CAT	GAG	TTC	CAG	988
Asp	Glu	Lou	Asp	Cys	Ala	Pro	Pro	Thr	Cys	Gly	Ala	His	Glu	Phe	Gln	
	185					190					195					
TGC	AGC	ACC	TCC	TCC	TCC	ATC	CCC	ATC	AGC	TCC	GTA	TGC	GAC	GAT	GAT	1036
Cys	Sor	Thr	Sor	Sor	Cys	Ile	Pro	Ile	Sor	Trp	Val	Cys	Asp	Asp	Asp	
200					205					210					215	
CCA	GAC	TCC	TCC	GAC	CAA	TGT	GAT	GAG	TCC	CTG	GAG	CAG	TGT	CCC	CGT	1084
Ala	Asp	Cys	Sor	Asp	Gln	Sor	Asp	Glu	Sor	Lou	Glu	Gln	Cys	Gly	Arg	
				220					225					230		
CAG	CCA	CTC	ATA	CAC	ACC	AAG	TGT	CCA	CCC	AGC	CAA	ATC	CAG	TCC	CCC	1132
Gln	Pro	Val	Ile	His	Thr	Lys	Cys	Pro	Ala	Sor	Glu	Ile	Gln	Cys	Gly	
			235					240					245			
TCT	CCC	GAG	TCC	ATC	CAT	AAG	AAG	TCC	CGA	TGT	GAT	CCC	GAC	CCT	GAC	1180
Sor	Gly	Glu	Cys	Ile	His	Lys	Lys	Trp	Arg	Cys	Asp	Gly	Asp	Pro	Asp	
		250					255					260				
TGC	AAG	GAT	CCC	AGT	GAT	GAG	CTC	AAC	TGT	CCC	TGT	CGA	ACT	TCC	CGA	1228
Cys	Lys	Asp	Gly	Sor	Asp	Glu	Val	Asn	Cys	Pro	Sor	Arg	Thr	Cys	Arg	
	265					270					275					
CCT	GAC	CAA	TTT	CAA	TGT	GAG	GAT	CCC	AGC	TCC	ATC	CAT	CCC	AGC	AGC	1276
Pro	Asp	Gln	Phe	Glu	Cys	Glu	Asp	Gly	Sor	Cys	Ile	His	Gly	Sor	Arg	
280					285					290					295	
CAG	TGT	AAT	CGT	ATC	CGA	GAC	TGT	CTC	GAT	CGT	TCC	GAT	CAA	CTC	AAC	1324
Gln	Cys	Asn	Gly	Ile	Arg	Asp	Cys	Val	Asp	Gly	Sor	Asp	Glu	Val	Asn	
			300					305					310			
TGC	AAA	AAT	CTC	AAT	CAG	TCC	TTC	CCC	CCT	CGA	AAA	TTC	AAG	TCC	AGA	1372
Cys	Lys	Asn	Val	Asn	Gln	Cys	Lou	Gly	Pro	Gly	Lys	Phe	Lys	Cys	Arg	
			315				320					325				
AGT	CGA	CAA	TCC	ATA	GAT	ATC	AGC	AAA	GTA	TGT	AAC	CAG	GAG	CAG	GAC	1420
Sor	Gly	Glu	Cys	Ile	Asp	Ile	Sor	Lys	Val	Cys	Asn	Gln	Glu	Gln	Asp	
		330					335					340				
TGC	AGC	GAC	TCC	AGT	GAT	GAG	CCC	CTG	AAA	GAG	TGT	CAT	ATA	AAC	CAA	1468
Cys	Arg	Asp	Trp	Sor	Asp	Glu	Pro	Lou	Lys	Glu	Cys	His	Ile	Asn	Glu	
	345					350					355					
TGC	TTC	GTA	AAT	AAT	CGT	CGA	TGT	TGT	CAT	ATC	TCC	AAA	GAC	CTA	GTT	1516
Cys	Lou	Val	Asn	Asn	Gly	Gly	Cys	Sor	His	Ile	Cys	Lys	Asp	Lou	Val	
360					365					370					375	
ATA	CCC	TAC	GAG	TGT	GAC	TGT	CGA	GCT	CCC	TTT	CAA	CTG	ATA	GAT	AGC	1564
Ile	Gly	Tyr	Glu	Cys	Asp	Cys	Ala	Ala	Gly	Phe	Glu	Lou	Ile	Asp	Arg	
			380						385					390		
AAA	ACC	TGT	CGA	GAT	ATT	GAT	CAA	TCC	CAA	AAT	CCA	CGA	ATC	TCC	AGT	1612
Lys	Thr	Cys	Gly	Asp	Ile	Asp	Glu	Cys	Gln	Asn	Pro	Gly	Ile	Cys	Sor	
			395				400						405			

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CAA ATT TGT ATC AAC TTA AAA CCC GGT TAC AAG TGT GAA TGT AGT GGT Gln Ile Cys Ile Asn Leu Lys Gly Gly Tyr Lys Cys Glu Cys Ser Arg 410 415 420	1660
CCC TAT CAA ATG GAT GTT GCT ACT CCC GTG TGC AAG GCA GTA CCC AAA Ala Tyr Glu Met Asp Leu Ala Thr Gly Val Cys Lys Ala Val Gly Lys 425 430 435	1708
GAG CCA AGT GTG ATC TTC ACT AAT GGA AGA GAC ATC AGC AAG ATT CCC Glu Pro Ser Leu Ile Phe Thr Asn Arg Arg Asp Ile Arg Lys Ile Gly 440 445 450 455	1756
TTA GAG AGC AAA GAA TAT ATC CAA CTA GTT GAA CAG CTA AGA AAC ACT Leu Glu Arg Lys Glu Tyr Ile Gln Leu Val Glu Gln Leu Arg Asn Thr 460 465 470	1804
GTG GCT CTC GAT GCT GAC ATT GCT GCC CAG AAA CTA TTC TCG CCC GAT Val Ala Leu Asp Ala Asp Ile Ala Ala Gln Lys Leu Phe Trp Ala Asp 475 480 485	1852
CTA AGC CAA AAG GCT ATC TTC AGT GCC TCA ATT GAT GAC AAG GTT GGT Leu Ser Glu Lys Ala Ile Phe Ser Ala Ser Ile Asp Asp Lys Val Gly 490 495 500	1900
AGA CAT GTT AAA ATG ATC GAC AAT GTC TAT AAT CCT GCA CCC ATT GCT Arg His Val Lys Met Ile Asp Asn Val Tyr Asn Pro Ala Ala Ile Ala 505 510 515	1948
GTG GAT TGG GTG TAC AAG ACC ATC TAC TGG ACT GAT GCG GCT TCT AAG Val Asp Trp Val Tyr Lys Thr Ile Tyr Trp Thr Asp Ala Ala Ser Lys 520 525 530 535	1996
ACT ATT TCA GTA GCT ACC CTA GAT GGA ACC AAG AGC AAG TTC CTC TTT Thr Ile Ser Val Ala Thr Leu Asp Gly Thr Lys Arg Lys Phe Leu Phe 540 545 550	2044
AAC TCT GAC TTG CGA GAG CCT GCC TCC ATA GCT GTG GAC CCA CTC TCT Asn Ser Asp Leu Arg Glu Pro Ala Ser Ile Ala Val Asp Pro Leu Ser 555 560 565	2092
CCC TTT GTT TAC TGG TCA GAC TGG GGT GAA CCA GCT AAA ATA GAA AAA Gly Phe Val Tyr Trp Ser Asp Trp Gly Glu Pro Ala Lys Ile Glu Lys 570 575 580	2140
GCA GGA ATG AAT GGA TTC GAT AGA GGT CCA CTC GTG ACA GCG GAT ATC Ala Gly Met Asn Gly Phe Asp Arg Arg Pro Leu Val Thr Ala Asp Ile 585 590 595	2188
GAG TGG CCT AAC GGA ATT ACA GTT GAC GTT ATA AAA AGT CCC CTC TAT Gln Trp Pro Asn Gly Ile Thr Leu Asp Leu Ile Lys Ser Arg Leu Tyr 600 605 610 615	2236
TGG CTT GAT TCT AAG TTG CAC ATG TTA TCC AGC GTG GAC TTG AAT GCG Trp Leu Asp Ser Lys Leu His Met Leu Ser Ser Val Asp Leu Asn Gly 620 625 630	2284
CAA GAT CGT AGC ATA GTA CTA AAG TCT CTC GAG TTC CTA GCT CAT CCT Gln Asp Arg Arg Ile Val Leu Lys Ser Leu Glu Phe Leu Ala His Pro 635 640 645	2332
CTT GCA CTA ACA ATA TTT GAG GAT CGT GTC TAC TGG ATA GAT GCG GAA Leu Ala Leu Thr Ile Phe Glu Asp Arg Val Tyr Trp Ile Asp Gly Glu 650 655 660	2380

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AAU GAA GCA GTC TAT COT CCC AAT AAA TTC ACT CGA TCA GAG CAT CCC Aon Glu Ala Val Tyr Gly Ala Aon Lys Phe Thr Gly Ser Glu His Ala 665 670 675	2428
ACT GTA GTC AAC AAC GTC AAT GAT CCC GAA GAC ATC ATT GTC TAT CAT Thr Lou Val Aon Aon Lou Aon Asp Ala Gln Asp Ile Ile Val Tyr His 680 685 690 695	2476
GAA CTT GTA CAG CCA TCA COT AAA AAT TCG TGT GAA GAA GAC ATC GAG Glu Lou Val Gln Pro Ser Gly Lys Aon Trp Cys Glu Glu Asp Met Glu 700 705 710	2524
AAT CGA CGA TGT GAA TAC GTA TGC CTC CCA GCA CCA CAG ATT AAT GAT Aon Gly Gly Cys Glu Tyr Lou Cys Lou Pro Ala Pro Gln Ile Aon Asp 715 720 725	2572
CAC TGT CCA AAA TAT ACC TGT TCC TGT CCC AGT CCG TAC AAT GTA GAG His Ser Pro Lys Tyr Thr Cys Ser Cys Pro Ser Gly Tyr Aon Val Glu 730 735 740	2620
GAA AAT CCG CGA GAC TGT CAA AGT ACT GCA ACT ACT GTC ACT TAC AGT Glu Aon Gly Arg Asp Cys Gln Ser Thr Ala Thr Thr Val Thr Tyr Ser 745 750 755	2668
GAG ACA AAA GAT ACC AAC ACA ACA GAA ATT TCA GCA ACT AGT CGA CTA Glu Thr Lys Asp Thr Aon Thr Thr Glu Ile Ser Ala Thr Ser Gly Lou 760 765 770 775	2716
GTT CCT CGA CCG ATC AAT GTC ACC ACA GCA GTA TCA GAG GTC AGT GTT Val Pro Gly Gly Ile Aon Val Thr Thr Ala Val Ser Glu Val Ser Val 780 785 790	2764
CCC CCA AAA CCG ACT TGT CCC GCA TCG CCC ATT CTT CCT CTC TTG CTC Pro Pro Lys Gly Thr Ser Ala Ala Trp Ala Ile Lou Pro Lou Lou Lou 795 800 805	2812
TTA GTC ATC GCA GCA GTA COT CCC TAC TTG ATC TCG CCG AAT TCG CAA Lou Val Met Ala Ala Val Gly Gly Tyr Lou Met Trp Arg Aon Trp Gln 810 815 820	2860
CAC AAC AAC ATC AAA AGC ATG AAC TTT GAG AAT CCT GTC TAC TTG AAA His Lys Aon Met Lys Ser Met Aon Phe Asp Aon Pro Val Tyr Lou Lys 825 830 835	2908
ACC ACT GAA GAG GAC CTC TCC ATA GAC ATT COT AGA CAC AGT CCT TCT Thr Thr Glu Glu Asp Lou Ser Ile Asp Ile Gly Arg His Ser Ala Ser 840 845 850 855	2956
GTT CGA CAC ACC TAC CCA CCA ATA TCA GTT GTA AGC ACA GAT GAT GAT Val Gly His Thr Tyr Pro Ala Ile Ser Val Val Ser Thr Asp Asp Asp 860 865 870	3004
CTA GCT TGACTTCTGT GACAAATGTT GACCTTTGAG GTCTAAACAA ATAATACCCC Lou Ala	3060
CGTCGGAATG GTAACCGAGC CAGCAGCTGA AGTCTCTTTT TCTTCCTCTC GGCTGGAAGA	3120
ACATCAAGAT ACCTTTGCGT CGATCAAGCT TGCTGTACTT GACCGTTTTT ATATTACTTT	3180
TGTAAATATT CTTGTCCACA TTCTACTTCA GCTTTGGATG TCGTTACCGA GTATCTGTAA	3240
CCCTTGAATT TCTAGACAGT ATTGCCACCT CTGGCCAAAT ATGCACTTTC CCTAGAAAGC	3300

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CATATCCAG CAGTGAACT TGTGCTATAG TGTATACCAC CTGTACATAC ATTGTATAGG 3360
 CCATCTGTAA ATATCCCAGA GAACAATCAC TATTCTTAAG CACTTTGAAA ATATTTCTAT 3420
 GTAAATTATT GTAAACTTTT TCAATGGTTG GGACAATGGC AATAGGACAA AACGGGTTAC 3480
 TAAGATGAAA TTGCCAAAAA AATTTATAAA CTAATTTTGG TACGTATGAA TGATATCTTT 3540
 GACCTCAATG GAGGTTTGCA AAGACTGAOT GTTCAAAC TA CTGTACATTT TTTTCAAGT 3600
 GCTAAAAAAT TAAACCAAGC AGCTTAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAA 3656

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 873 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Thr Ser Ala Leu Trp Ala Val Trp Leu Leu Leu Ala Leu Cys
 1 5 10 15
 Trp Ala Pro Arg Glu Ser Gly Ala Thr Gly Thr Gly Arg Lys Ala Lys
 20 25 30
 Cys Glu Pro Ser Gln Phe Gln Cys Thr Asn Gly Arg Cys Ile Thr Leu
 35 40 45
 Leu Trp Lys Cys Asp Gly Asp Glu Asp Cys Val Asp Gly Ser Asp Glu
 50 55 60
 Lys Asn Cys Val Lys Lys Thr Cys Ala Glu Ser Asp Phe Val Cys Asn
 65 70 75 80
 Asn Gly Gln Cys Val Pro Ser Arg Trp Lys Cys Asp Gly Asp Pro Asp
 85 90 95
 Cys Glu Asp Gly Ser Asp Glu Ser Pro Glu Gln Cys His Met Arg Thr
 100 105 110
 Cys Arg Ile His Glu Ile Ser Cys Gly Ala His Ser Thr Gln Cys Ile
 115 120 125
 Pro Val Ser Trp Arg Cys Asp Gly Glu Asn Asp Cys Asp Ser Gly Glu
 130 135 140
 Asp Glu Glu Asn Cys Gly Asn Ile Thr Cys Ser Pro Asp Glu Phe Thr
 145 150 155 160
 Cys Ser Ser Gly Arg Cys Ile Ser Arg Asn Phe Val Cys Asn Gly Gln
 165 170 175
 Asp Asp Cys Ser Asp Gly Ser Asp Glu Leu Asp Cys Ala Pro Pro Thr
 180 185 190

67

Cys Gly Ala His Glu Phe Gln Cys Ser Thr Ser Ser Cys Ile Pro Ile
 195 200 205
 Ser Trp Val Cys Asp Asp Asp Ala Asp Cys Ser Asp Gln Ser Asp Glu
 210 215 220
 Ser Leu Glu Gln Cys Gly Arg Gln Pro Val Ile His Thr Lys Cys Pro
 225 230 235 240
 Ala Ser Glu Ile Gln Cys Gly Ser Gly Glu Cys Ile His Lys Lys Trp
 245 250 255
 Arg Cys Asp Gly Asp Pro Asp Cys Lys Asp Gly Ser Asp Glu Val Asn
 260 265 270
 Cys Pro Ser Arg Thr Cys Arg Pro Asp Gln Phe Glu Cys Glu Asp Gly
 275 280 285
 Ser Cys Ile His Gly Ser Arg Gln Cys Asn Gly Ile Arg Asp Cys Val
 290 295 300
 Asp Gly Ser Asp Glu Val Asn Cys Lys Asn Val Asn Gln Cys Leu Gly
 305 310 315 320
 Pro Gly Lys Phe Lys Cys Arg Ser Gly Glu Cys Ile Asp Ile Ser Lys
 325 330 335
 Val Cys Asn Gln Glu Gln Asp Cys Arg Asp Trp Ser Asp Glu Pro Leu
 340 345 350
 Lys Glu Cys His Ile Asn Glu Cys Leu Val Asn Asn Gly Gly Cys Ser
 355 360 365
 His Ile Cys Lys Asp Leu Val Ile Gly Tyr Glu Cys Asp Cys Ala Ala
 370 375 380
 Gly Phe Glu Leu Ile Asp Arg Lys Thr Cys Gly Asp Ile Asp Glu Cys
 385 390 395 400
 Gln Asn Pro Gly Ile Cys Ser Gln Ile Cys Ile Asn Leu Lys Gly Gly
 405 410 415
 Tyr Lys Cys Glu Cys Ser Arg Ala Tyr Gln Met Asp Leu Ala Thr Gly
 420 425 430
 Val Cys Lys Ala Val Gly Lys Glu Pro Ser Leu Ile Phe Thr Asn Arg
 435 440 445
 Arg Asp Ile Arg Lys Ile Gly Leu Glu Arg Lys Glu Tyr Ile Gln Leu
 450 455 460
 Val Glu Gln Leu Arg Asn Thr Val Ala Leu Asp Ala Asp Ile Ala Ala
 465 470 475 480
 Gln Lys Leu Phe Trp Ala Asp Leu Ser Gln Lys Ala Ile Phe Ser Ala
 485 490 495
 Ser Ile Asp Asp Lys Val Gly Arg His Val Lys Met Ile Asp Asn Val
 500 505 510
 Tyr Asn Pro Ala Ala Ile Ala Val Asp Trp Val Tyr Lys Thr Ile Tyr
 515 520 525

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Trp Thr Asp Ala Ala Ser Lys Thr Ile Ser Val Ala Thr Leu Asp Gly
 530 535 540
 Thr Lys Arg Lys Phe Leu Phe Asn Ser Asp Leu Arg Glu Pro Ala Ser
 545 550 555 560
 Ile Ala Val Asp Pro Leu Ser Gly Phe Val Tyr Trp Ser Asp Trp Gly
 565 570 575
 Glu Pro Ala Lys Ile Glu Lys Ala Gly Met Asn Gly Phe Asp Arg Arg
 580 585 590
 Pro Leu Val Thr Ala Asp Ile Gln Trp Pro Asn Gly Ile Thr Leu Asp
 595 600 605
 Leu Ile Lys Ser Arg Leu Tyr Trp Leu Asp Ser Lys Leu His Met Leu
 610 615 620
 Ser Ser Val Asp Leu Asn Gly Gln Asp Arg Arg Ile Val Leu Lys Ser
 625 630 635 640
 Leu Glu Phe Leu Ala His Pro Leu Ala Leu Thr Ile Phe Glu Asp Arg
 645 650 655
 Val Tyr Trp Ile Asp Gly Glu Asn Glu Ala Val Tyr Gly Ala Asn Lys
 660 665 670
 Phe Thr Gly Ser Glu His Ala Thr Leu Val Asn Asn Leu Asn Asp Ala
 675 680 685
 Gln Asp Ile Ile Val Tyr His Glu Leu Val Gln Pro Ser Gly Lys Asn
 690 695 700
 Trp Cys Glu Glu Asp Met Glu Asn Gly Gly Cys Glu Tyr Leu Cys Leu
 705 710 715 720
 Pro Ala Pro Gln Ile Asn Asp His Ser Pro Lys Tyr Thr Cys Ser Cys
 725 730 735
 Pro Ser Gly Tyr Asn Val Glu Glu Asn Gly Arg Asp Cys Gln Ser Thr
 740 745 750
 Ala Thr Thr Val Thr Tyr Ser Glu Thr Lys Asp Thr Asn Thr Thr Glu
 755 760 765
 Ile Ser Ala Thr Ser Gly Leu Val Pro Gly Gly Ile Asn Val Thr Thr
 770 775 780
 Ala Val Ser Glu Val Ser Val Pro Pro Lys Gly Thr Ser Ala Ala Trp
 785 790 795 800
 Ala Ile Leu Pro Leu Leu Leu Leu Val Met Ala Ala Val Gly Gly Tyr
 805 810 815
 Leu Met Trp Arg Asn Trp Gln His Lys Asn Met Lys Ser Met Asn Phe
 820 825 830
 Asp Asn Pro Val Tyr Leu Lys Thr Thr Glu Glu Asp Leu Ser Ile Asp
 835 840 845

Ile Gly Arg His Ser Ala Ser Val Gly His Thr Tyr Pro Ala Ile Ser
850 855 860

Val Val Ser Thr Asp Asp Asp Leu Ala
865 870

(2) INFORMATION FOR SEQ ID NO:3:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9592 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTCGCTA GCATCATCAA TAATATACCT TATTTTCGAT TCAAGCCCAAT ATGATAATGA	60
CCCCCTCGAG TTTCTGACCT CCCCCCCCCC GTCCGAACCG CCGCTCTGAC GTAGTAGTGT	120
CCCCGAAGTC TCATCTTCCA AGTCTCCCGC AACCATGTA ACCGACCGAT GTCCAAAAAG	180
TCACCTTTTT CGTCTCCCGC CGTCTACACA CCAAGTCACA ATTTTCCCGC CGTTTTAGGC	240
CGATGTTGTA GTAAATTTCG CCGTAACCGA GTAAATTTTC GCCATTTTCC CCGGAAAACT	300
GAATAAGAGC AAGTCAAAATC TGAATAAATT TGTCTTACTC ATAGCCCGTA ATATTCTCTT	360
ACCGAGATCA GCCTCCAGCT CATTACATTA CTTACCGTAA ATCCCGCCCG TCGCTGACCG	420
CCCAACGACC CCGCCCGATT GACGTCAATA ATGACGTATC TTCCCATAGT AACGCCAATA	480
CGGACTTTTC ATTGACCTCA ATCCCTCGAG TATTTACCGT AACTGCCCCA CTTCGCAGTA	540
CATCAAGTGT ATCATATCCC AAGTACCGCC CATTTCAGC TCAATGACCG TAAATCGCCC	600
GCCTCCGATT ATCCCGAGTA CATGACCTTA TCGGACTTTC GTACTTCGCA GTACATCTAC	660
GTATTAGTCA TCGCTATTAC CATGCTGATC CGCTTTTCCG AGTACATCAA TCGCGCTCGA	720
TACCGCTTTC ACTCAGCGCG ATTTCGAAGT GTCCACCCCA TTGACGTCAA TCGGAGTTTC	780
TTTTCCGACC AAAATCAAGC CGACTTTCCA AAATGTGCTA ACAACTCCCG CCCATTGACG	840
CAATCGCGCG GTAGCGCTGT ACCGTCCGAG GTGTATATAA GCAGAGCTCT CTGCTAACT	900
AGAGAACCCA CTGCTTAACT CGCTTATGCA AATTAAATAG ACTCACTATA CGGAGACCCA	960
AGCTTCTCTG CCGCCCGCGG GTCCCGGTGC TCGTACCGCG CTCTCTCCGT TCTGTCTCTT	1020
CTTCTCTCTT CCGCTCCCCA CCGCTCTCC GTTCCCTCTT CTCCCCTTCC CTCCCCTCTT	1080
CTGCAGCGCC TCATTATTTT TCTCCCGCCA CTTCCGCTTC CACTGCTGCT GCAGCCCGGG	1140
GAGGTGGCTG CGTCCGCTCG GAGGAGACTG TCCAAGTTGT ACGCGAGCGG GTCCCTCTTT	1200
CTTCCCCGCT CCGTTCCCCA GCCAAGTGGT TCCCCCTCTT CTCCCCCTTT CCGCTCCGAG	1260

CCCCCACTTT	CTTCTCTTTT	CGAAGCCCT	CGTAACCTTGT	CGTCGCGACC	GAACCGCCGC	1320
CCCGCCGCGC	CCCGCCGCGC	CATCCAGCCG	CGCACCATCG	CGACGTCGCG	GCTCTCGCGC	1380
GTCTCGCTCG	TGCTCGCGCT	GTCTCGCGCG	CCCCCGGAGA	CGCGCGCCAC	CGGAACCGCG	1440
AGAAAAGCCA	AATGTGAACC	CTCCCAATTC	CAGTCACAAA	ATGTCTCGTG	TATTACCGTG	1500
TTGTGGAAT	GTGATCGCGA	TGAAGACTGT	GTTCAGCGCA	GTGATGAAA	GAAGTGTGTA	1560
AAGAAGAGCT	GTCTCGAATC	TGACTTCGTG	TGCAACAAAT	CGCAGTGTGT	TCCGAGCCGA	1620
TGGAAGTGTG	ATCGAGATCG	TGACTCGGAA	GATGTTTCA	ATGAAGCGCG	AGAACAGTGC	1680
CATATGAGAA	CATCGCCCAT	ACATGAAATC	AGCTGTGCGG	CGCATTCATC	TGAGTGTATC	1740
CCAGTGTCTT	CGAGATGTGA	TGCTGAAAT	GATTGTGACA	GTGAGAGAAG	TGAAGAAATC	1800
TGTGCGAATA	TAAATGTATC	TCCGAGCGAG	TTCACTCTCT	CGAGTGGCGG	CTGATCTTCC	1860
AGGAAGTTTG	TATCGAATCG	CGAGGATGAC	TGCAGCGATG	CGAGTGAATG	GCTCGAGTGT	1920
CCCCCGCCAA	CGTGTGCGCG	CGATGAGTTC	CAGTCAGCGA	GCTGCTCGTG	CATCGCCATC	1980
AGCTCGGTAT	CGGAGGATGA	TGCAAGCTGC	TCCGAGCAAT	CTGATGAGTC	GCTCGAGCGG	2040
TGTGCGCGTG	AGCGAGTCAT	ACAGACCAAG	TGTCCAGCGA	CGGAATGCGA	GTGCGGCTGT	2100
CGCGAGTGA	TGCATAAGAA	GTGCGGATGT	GATGCGGACC	CTGACTGCAA	CGATCGCAGT	2160
GATGAGCTGA	ACTGTGCGTG	TGGAAGTTGC	CGAGCTGACC	AATTTGAATG	TGAGGATGCG	2220
AGCTGCAATC	ATGCGAGCGG	CGAGTGTAA	CGTATCGGAG	ACTGTGTGGA	TGCTTCCGAT	2280
GAAGTCAACT	CGAAAATGT	CAATCAGTGC	TTGCGCGCTG	GAATTTCAA	GTGAGAAAGT	2340
CGAGAAATGA	TAGATATGAG	CAAGATATGT	AAGCAGGAGC	AGAGTGCAG	CGAGTGCAGT	2400
GATGAGCGCG	TGAAGAGTGC	TGATATAAAG	GAATGCTTGC	TAAATAAATG	TGATGTCTCT	2460
CATATCTGCA	AAGAGCTAGT	TATAGCGTAC	CAGTGTGACT	GTGAGCTGCG	GTTTGAACTG	2520
ATAGATAGGA	AAAGCTGTGC	AGATATTGAT	GAATGCGAAA	ATCCAGGAAT	CTGAGTCAA	2580
AATTTATATCA	ACTTAAAGCG	CGGTTACAG	TGTGAATGTA	GTGCTGCGTA	TCAATGCGAT	2640
CTTCTACTG	CGGTGTGCAA	CGGAGTAGCG	AAGAGCGCAA	GTCTGATCTT	CACTAATCGA	2700
AGAGACATGA	CGAAGATTCG	GTTAGAGAGG	AAGAAATATA	TCCAACTAGT	TGAACAGCTA	2760
AGAAACACTG	TGCTCTGCGA	TGCTGACATT	GCTGCGCGAG	AAGTATTGTC	CGCGGATCTA	2820
AGCCAAAAGG	CTATCTTCAG	TGCTCAATTT	GATGACAAGG	TTGCTAGACA	TGTTAAATG	2880
ATGAGCAATG	TGTATAATTC	TGAGCGCAAT	GCTGTTGATT	CGGTGTACAA	GACCATCTAC	2940
TGAGTGAATG	CGGCTTCTAA	GACTATTTC	GTAGCTACCC	TAGATGGAAC	CAAGAGGAAG	3000
TTCTCTTTTA	ACTGTGACTT	CGGAGAGCGT	CGCTCCATAG	CTGTGAGCCC	ACTGTCTGCG	3060
TTTGTTTACT	GCTCAGACTG	CGGTGAACCA	GCTAAATATG	AAAAAGCAGG	AATGAATGGA	3120

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TTGAATGCGC	AAGATGCTAG	GATACTACTA	AAGTCTCTCC	AGTTGCTAGC	TCATCCTCTT	3300
CCACTAACAA	TATTTGAGCA	TCGTGTCTAC	TGATATAGAT	GGAAAAATGA	AGCACTCTAT	3360
CGTGCCAAAT	AATTCAGTCC	ATCAGAGCAT	CGCACTCTAG	TCAACAACCT	GAATGATGCC	3420
CAAGACATCA	TTGTCTATCA	TGAAGTTGTA	CAGCCATCAG	GTAAAAATTC	GTGTGAAGAA	3480
GACATGAGAA	ATGAGGAGAT	TGAATACCTA	TGCGTCCGAG	CAGCAGAGAT	TAATGATCAG	3540
TCTGCAAAAT	ATACCTGTTT	CTGTCCCACT	CGGTACAAAT	TAGAGCAAAA	TGCGCGAGAC	3600
TGTCAAAATA	GTGCAACTAC	TGTGACTTAG	AGACAAAAGA	TAGCAACACA	ACAGAAATTT	3660
CAGCAACTAG	TGCACTAGTT	CCTGCAAGCA	TCAATGTGAC	CACAGCACTA	TCAGAGCTCA	3720
GTGTTCCGCC	AAAAGCGACT	TCTGCGGCAAT	CGGCAATTC	TGCTGTCTTC	GTCTTAGTGA	3780
TGCGAGCACT	AGGTGCGTAC	TTGATGTGCG	CGAATTCGCA	ACACAGCAAC	ATGAAAAGCA	3840
TGAACTTTGA	CAATCCTGTG	TACTTGAAAA	CCAGTGAAAG	CGACCTCTCC	ATAGACATTC	3900
GTAGACACAG	TGCTTCTGTT	CGACACAGCT	ACCCAGCAAT	ATCACTTGTG	AGCAGAGATG	3960
ATGATCTAGC	TTGACTTCTG	TGACAAATGT	TGACCTTTGA	GGTCTAAACA	AATAATACCC	4020
CGGTGCGAAT	CGTAAGCGAG	CGAGCAGCTG	AAGTCTCTTT	TTCTTCCCTT	CGGCTGGAAG	4080
AACATCAAGA	TACCTTTGCG	TGATCAAGC	TTGCTACCGA	CGTCCGATCC	ACTAGTAAGC	4140
CGCGCCAGTG	TGCTGGAATT	GTGAGATAT	CCATCAGACT	CGCGCCCGCG	CGGATCCAGA	4200
CATGATAGA	TACATTCATG	AGTTTGCAAC	AACCACAACT	AGAAATCAGT	CAAAAAATG	4260
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ACAAGTTAAC	AACAACAATT	GCATTCATTT	TATGTTTCAG	GTTCAGCGCG	AGGTGTGCGA	4380
CGTTTTTTCC	GATCCTCTAG	AGTCAAGCTG	CAGGCTGATC	TGGAAGGTGC	TGAGGTACGA	4440
TGAGACCGCG	ACCAGGTGCA	GAGCGTCCGA	GTGTGCGCGT	AAACATATTA	CGAACCAGCC	4500
TGTGATGCTG	GATGTGACCG	AGGAGCTGAG	CGCGCATCAC	TTGCTGCTCG	CCTGCACCGG	4560
CGCTGAGTTT	CGCTCTAGCG	ATGAAGATAC	AGATTGAGGT	ACTGAAATGT	GTGCGCGTGC	4620
GTTAAAGGTC	CGAAAGCAATA	TATAAGCTCG	CGGTCTTATG	TAGTTTTGTA	TCTGTTTTCC	4680
AGCAGCGCGC	CGCGCCATGA	GCACCAACTC	GTTTGATGGA	AGCATTGTGA	GCTCATATTT	4740
GACAAAGCGC	ATGCCCCCAT	CGCGCGCGGT	CGGTGAGAAAT	GTAATGCGCT	CCAGCATTCG	4800
TGCTGCGCGC	GTGCTGCGCG	CAAACTCTAC	TACCTTGACC	TAGGAGACCG	TGTCTGGAAC	4860
CGCGTTGAGC	ACTGCAGCGT	CGCGCGCGCG	TTCAAGCGGT	GCAGCCACCG	CGCGCGCGAT	4920
TGTGACTGAC	TTTGCTTTCC	TGAGCGCGCT	TGCAAGCAGT	GCAGCTTCCC	GTTCATCCGC	4980

CCGCCATGAC	AACTTCACCG	CTCTTTTCCG	ACAAATGCGT	TCTTTGACCG	CCCAAGTTAA	5040
TCTCCTTTCT	CAGCAGCTGT	TGATCTCCG	CCAGCAGCTT	TCTCCCTGCA	AGCCTTCCTC	5100
CCCTCCCAAT	CCGCTTTAAA	ACATAAATAA	AAAACCAAGC	TCTCTTTGCA	TTTCAATCAA	5160
CCAACTGTCT	TGCTGTCTTT	ATTAGCCCTT	TTTCCGCGCG	CGGTAGCCCG	CCGACCAAGC	5220
GTCTCCCTCG	TTAGCCCTCG	TGTCTATTTT	TTCCAGCAGC	TGCTAAAGCT	CAGTCTCGAT	5280
GTTCAGATAC	ATCCCAATAA	CGCCCTCTCT	CCGCTGAGCG	TAGCAGCACT	CCAGAGCTTC	5340
ATCTCCCGCG	GTCTCTTTCT	AGATGATGCA	GTCTAGCAGC	CAGCCTCTCG	CGTCTCTCTT	5400
AAAAATGTCT	TTCAATAGCA	AGCTCATTCG	CAGCCCGCAG	CGCTTCTCTT	AACTCTTTAC	5460
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CAGCAGCTCG	TATCCCTCTG	AGTTCCGAAA	TTTCTCATCT	AGCTTAGAAG	CAAACTCGTG	5640
CAAGAACTTG	CAGACCCCTT	TGTGACCTCG	AAATTTTCTC	ATGCATTCCT	CCATAATGAT	5700
CCCAATCCCG	CCAGCCCGCG	CGCCCTCTCG	CAAGATATTT	CTCCGATGAC	TAACTCATTA	5760
CTTCTCTTCC	AGCATGAGAT	CGTCATAGCG	CATTTTACAA	AAAGCCCGCG	CGAGCCTCTG	5820
AGACTCCCTT	ATAATGCTTC	CATCCCGCGC	AGCCCGCTAG	TTACCCCTGAC	AGATTTGCAAT	5880
TTCCCAAGCT	TTCACTTCAAG	ATCCCGCGAT	CATCTCTACC	TCCCGCGCGA	TCAAGAAAGC	5940
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ACCCAGCGCG	GTCCCGCGCT	AAATCAGACC	TATTAAGCGG	TCCAACTCTT	AGTTAAGAGA	6060
CGTCCAGCTG	CGCTCATCCC	TGAGCAGCGG	CGCCAGTTCC	TTAAGCATCT	CGCTGACTCG	6120
CATCTTTTCC	CTGACCAAAAT	CGCCAGAAAG	CGCTCTCGCG	CCAGCCGATA	CGAGTTCTTG	6180
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CAGCATATCT	CCTCCTTTCC	CGCTTTCCCG	CGCTTTTCCG	TGTAGCGCAG	TAGTCTCTCG	6360
TGCTCCAGAC	CGCCAGCGCT	CATCTCTTTC	CAGCTCGCGA	CGCTCTCTCT	CAGCCTAGTC	6420
TGCTCAGCGG	TAAAGCGCTG	CGCTCGCGCG	TGCTCTCTCG	CCAGCCTCGG	CTTCAAGCTG	6480
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TGTCTTCTTT	ATCATGCAAC	TCGTAGGACA	CGTGGCGGCA	CGGCTCTGCG	TCATTTTCCG	7020
CGAGGACCGC	TTTGGCTGCA	CGCGGAGGAT	GATCGGCGTG	TCGCTTGGCG	TATTGCGAAT	7080
CTTGCACCGC	CTGGCTGAAG	CGTTGCTCAC	TCGTCCGCGG	ACCAAGCGTT	TCGCGGAGAA	7140
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CGCATCTCCA	CGAGCGGCGC	CGCGCGCATC	TCGCGGAGCG	TTGGTCTCTG	CGCACGGCTG	7680
CGCATGATCG	TCGCTCTCTC	CTTCAAGGACC	CGGCTAGCGT	CGCGGGCTTG	CGTTACTGCT	7740
TAGCAGAATG	AATCAGCGAT	ACCGGAGCGA	ACGTGAAGCG	ACTGCTCTCT	CAAAACGTCT	7800
CGGACCTGAG	CAACAACATG	AATGGTCTTC	CGTTTCCGTC	TTTGGTAAGG	TCGCGAAACG	7860
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CGCGGACCGC	TCGCGCTTAT	CGGCTAACTA	TCGTCTTGAG	TCCAAACCGG	TAGACACCGA	8100
CTTATCGCCA	CTGGCAGCAG	CGACTGCTAA	CAGGATTAGC	AGAGCGAGGT	ATGTAGCGCG	8160
TGCTACAGAG	TTCTTGAAGT	CGTGGCGTAA	GTACGGCTAC	ACTAGAAAGG	CAGTATTTGG	8220
TATCTGCGCT	CTGCTGAAGC	CAGTTACCTT	CGGAAAAAGA	CTTGGTAAGT	CTTGAATCGG	8280
CAAAACAAAC	ACCGCTGCTA	CGGCTGCTTT	TTTTGTTTGC	AAGCAGCAGA	TTACGCGCAG	8340
AAAAAAAGGA	TCTCAAGAAAG	ATCCTTTGAT	CTTTCTAGCG	CGGTCTGAGG	CTCAGTGGAA	8400
CGAAAACCTA	CGTTAAGCGA	TTTTGGTCAAT	GAGATTATCA	AAAAAGATCT	TCACCTAGAT	8460
CGTTTTAAAT	TAAAAATGAA	CTTTTAAATC	AATCTAAAGT	ATATATGAGT	AAACTTGGTC	8520
TCACAGTTAC	CAATGCTTAA	TCAGTGAGCG	ACCTATCTCA	CGGATCTGTC	TATTTGCTTC	8580
ATCCATAGTT	CGCTGACTCC	CGGTGCTGTA	GATAACTAGG	ATACCGGAGG	CGTTACCATC	8640
TCGCGCGCAGT	GCTGCAATGA	TACCGCGAGA	CGCACGCTCA	CGGCTCCAG	ATTTATCAGC	8700

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AATAAACCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC	8760
CATCCAGTCT ATTAATTGTT GCGGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTTT	8820
GCGCAACGTT GTTGCCATTG CTGCAGGCAT CGTGCTGTCA CGCTCGTGGT TTGGTATGGC	8880
TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA TGATCCCCCA TGTGTGCAA	8940
AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT	9000
ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG	9060
CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTG TCGGGCGACC	9120
GAGTTGCTCT TCGCCGGCGT CAACACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA	9180
AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAACTC TCAAGGATCT TACCGCTGTT	9240
GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTACTTT	9300
CACCAGCGTT TCTGGGTGAG CAAAACAGG AAGGCAAAAT GCGCAAAAA AGGGAATAAG	9360
GGCGACACGG AAATGTTGAA TACTCATACT CTTCTTTTT CAATATTATT GAAGCATTTA	9420
TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT	9480
AGGGGTTCCG CGCACATTTC CCGAAAAGT GCCACCTGAC GTCTAAGAAA CCATTATTAT	9540
CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCC TTTGCTCTTC AA	9592

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TAGTAAATTT GGGC

14

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGTAAGATTT GGCC

14

75

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTGAAATCT GAAT

14

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAATAATTTT GTGT

14

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGTAATATTT GTCT

14

WHAT IS CLAIMED IS:

1. A recombinant viral vector comprising:
 - (a) the DNA of, or corresponding to, at least a portion of the genome of an adenovirus, which portion is capable of infecting a hepatic cell;
 - (b) a human *VLDL* receptor gene operatively linked to regulatory sequences directing its expression, said gene flanked by the DNA of (a) and capable of expression in the hepatic cell.
2. The vector according to claim 1 wherein said adenovirus DNA comprises the adenovirus 5' and 3' cis-elements necessary for replication and virion encapsidation in the absence of sequence encoding viral genes.
3. The vector according to claim 1 or 2 wherein said adenovirus genome has a deletion in all or a part of the E1 gene.
4. The vector according to any of claims 1 to 3 wherein said adenovirus genome has a deletion in all or a part of the E3 gene.
5. The vector according to any of claims 1 to 3 wherein said adenovirus genome comprising deletions in the DNA sequences of all or a portion of the adenovirus genes selected from the group consisting of the E2a gene, the E4 gene, the late genes L1 through L5, the intermediate genes IX and IV₁, and a combination thereof.
6. A mammalian hepatocyte which expresses a human *VLDL* receptor gene introduced therein through transduction of the vector of any one of claims 1 to 5.

7. Use of a recombinant viral vector for the manufacture of a medicament, said viral vector comprising:

(a) the DNA of, or corresponding to, at least a portion of the genome of an adenovirus, which portion is capable of infecting a hepatic cell;

(b) a human VLDL receptor gene operatively linked to regulatory sequences directing its expression, said gene flanked by the DNA of (a) and capable of expression in the hepatic cell.

8. The use according to claim 7 wherein the medicament is used for reducing cholesterol levels in familial hypercholesterolemia patients.

9. The use according to claim 7 wherein the medicament is used for reducing cholesterol levels in familial combined hyperlipidemia patients.

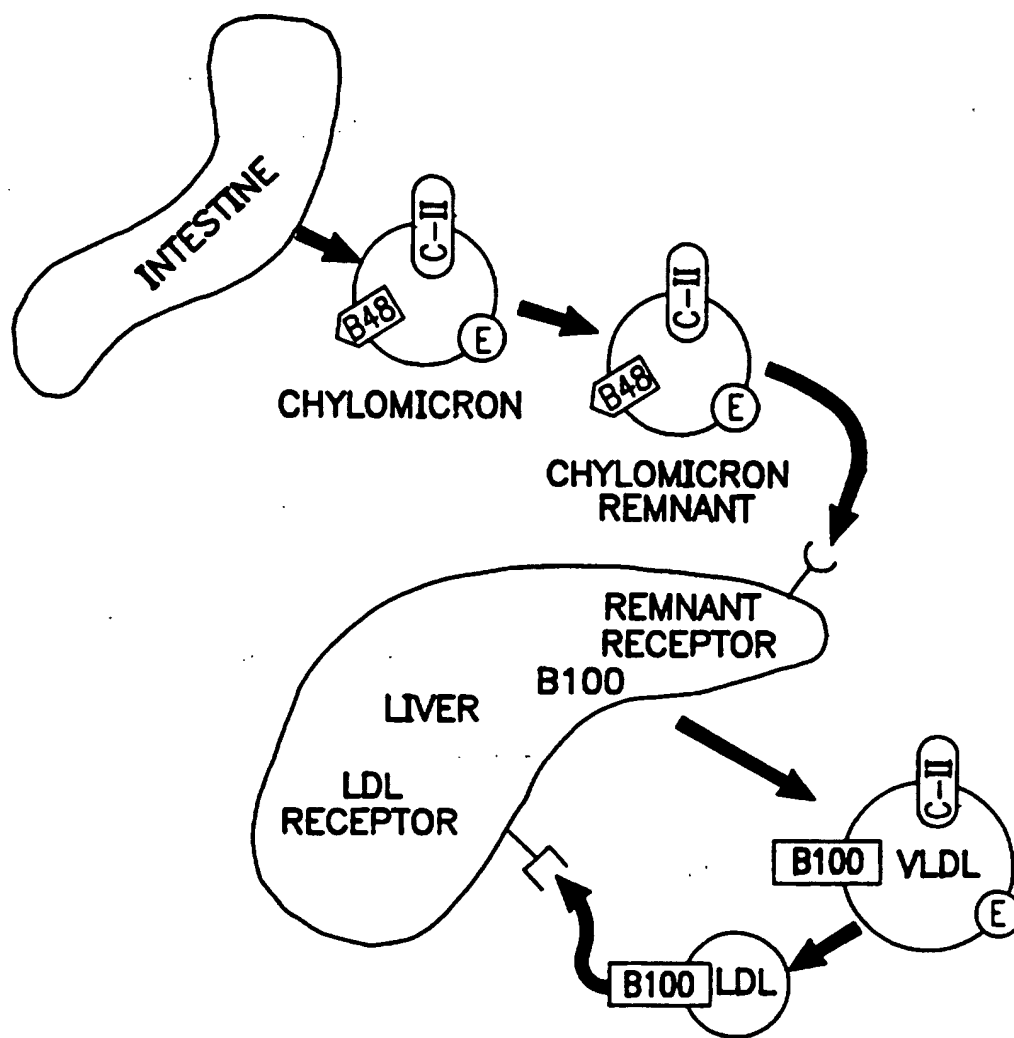


FIG. 1A

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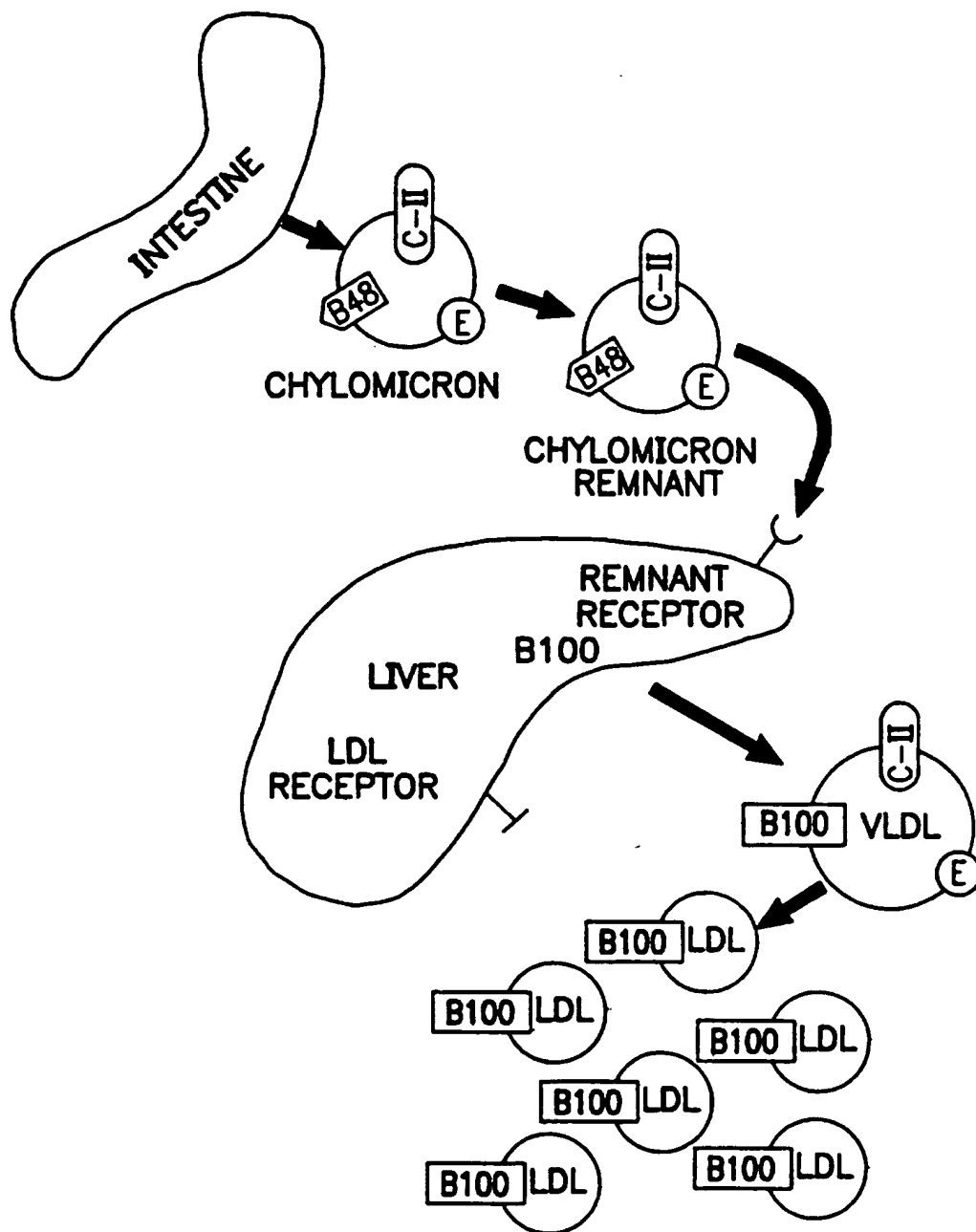


FIG. 1B

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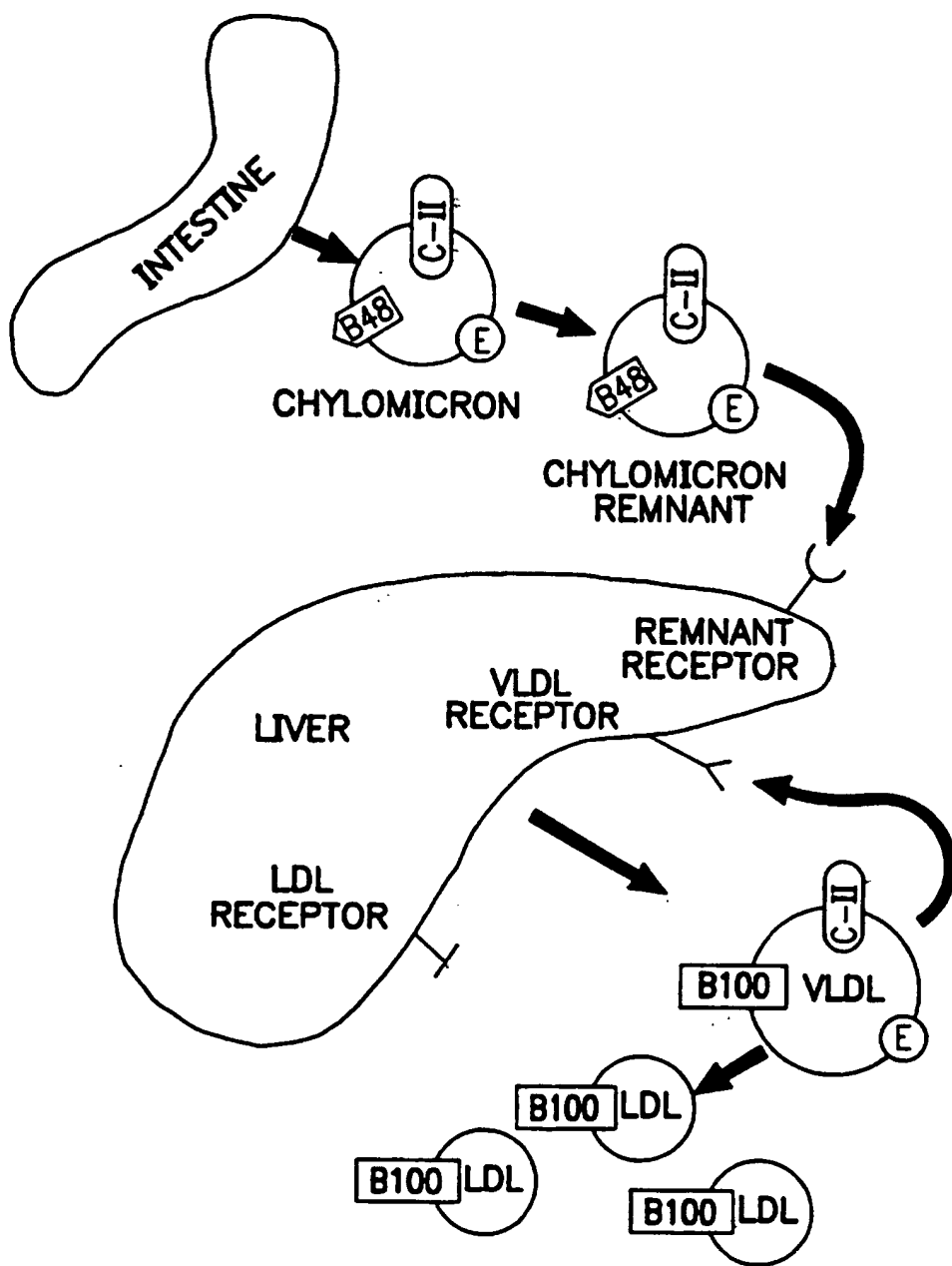


FIG. 1C

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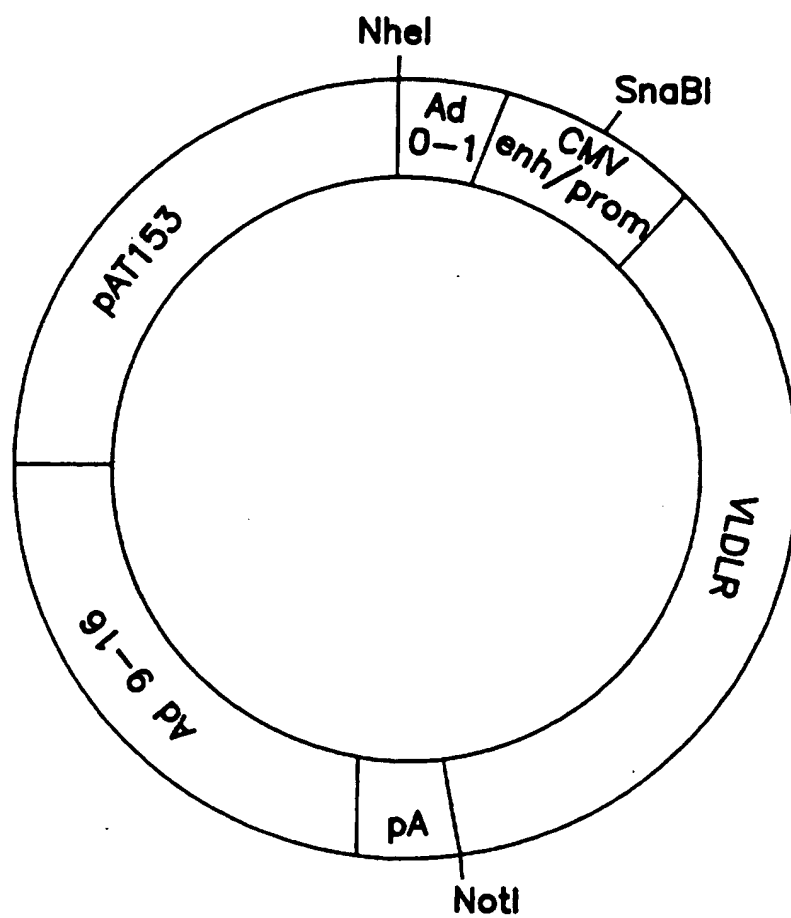


FIG. 2



FIG. 3

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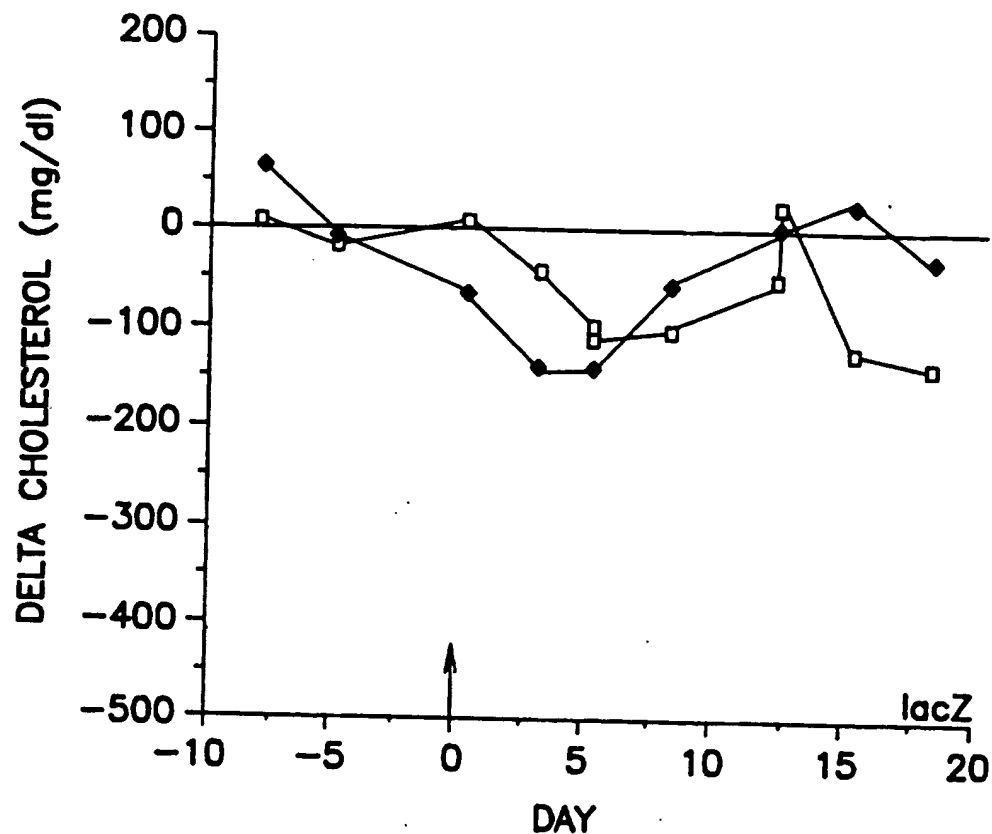


FIG. 4A

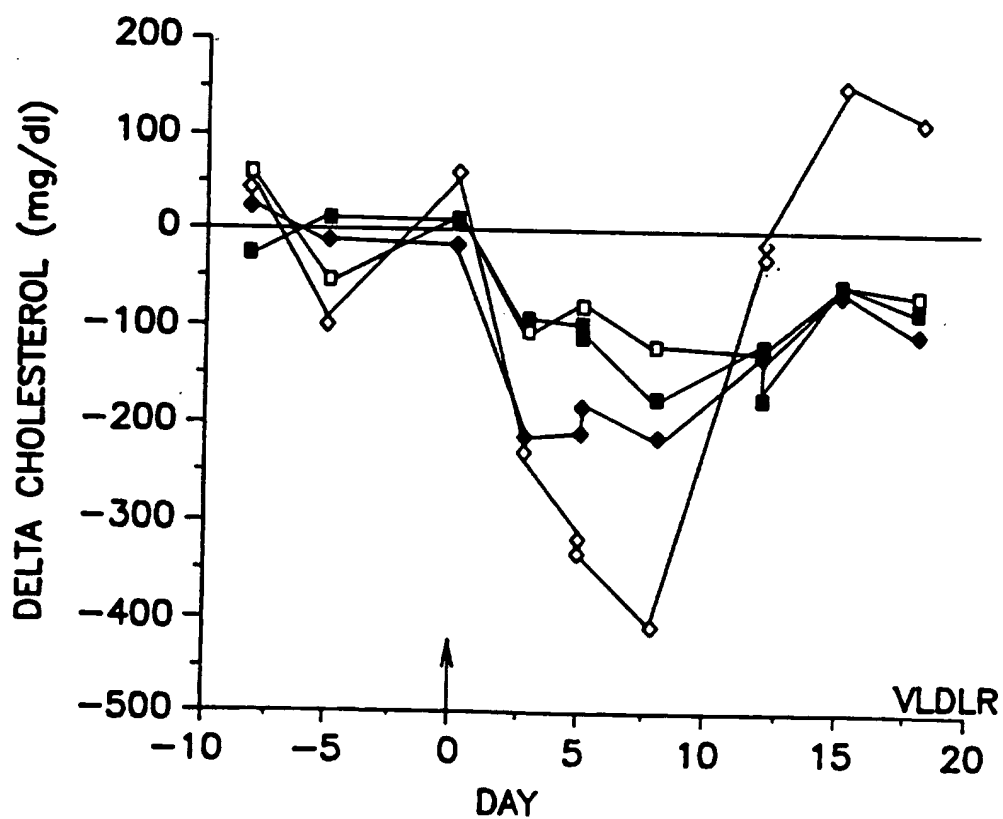


FIG. 4B

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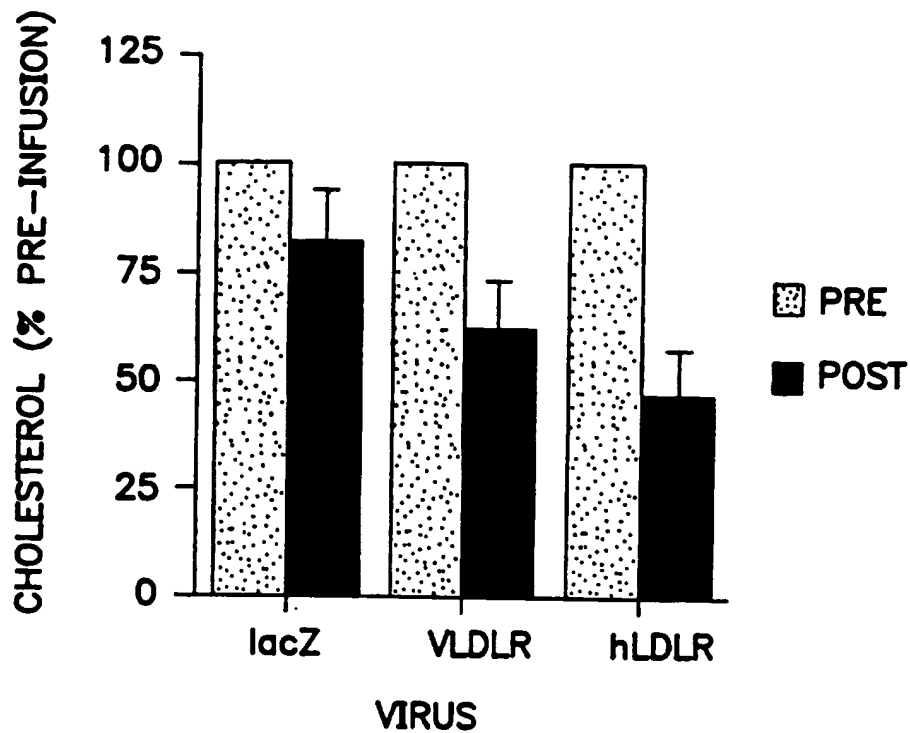


FIG. 5

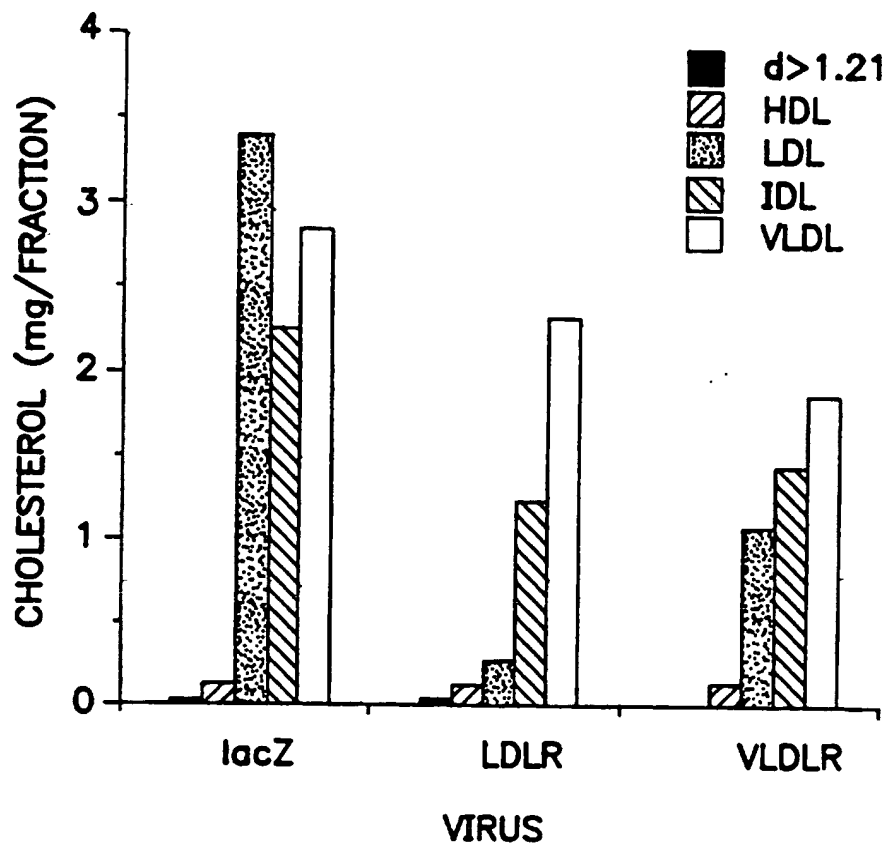


FIG. 6

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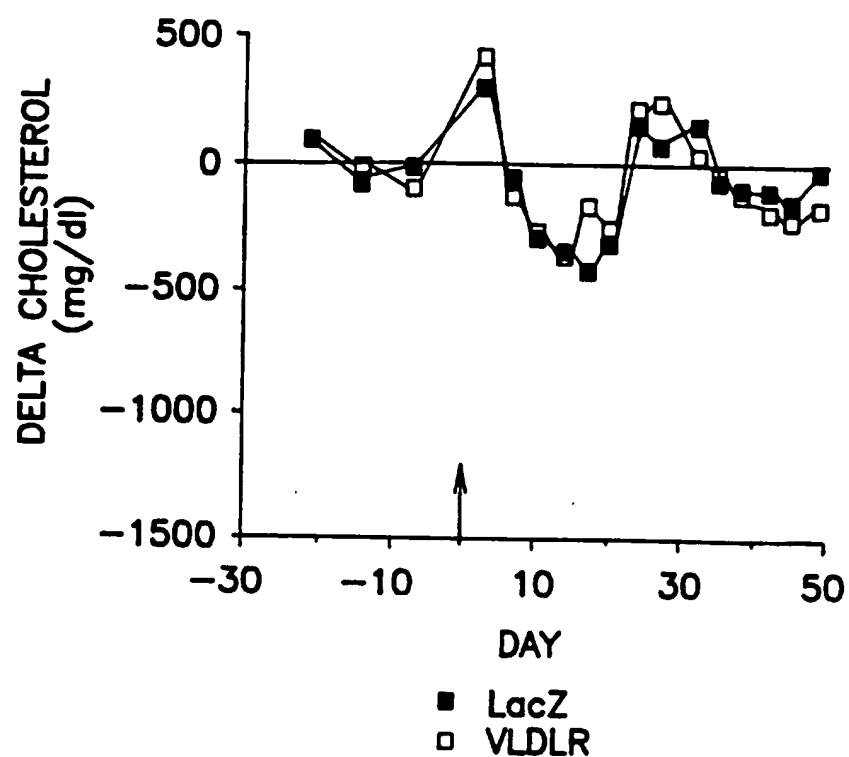


FIG. 7A

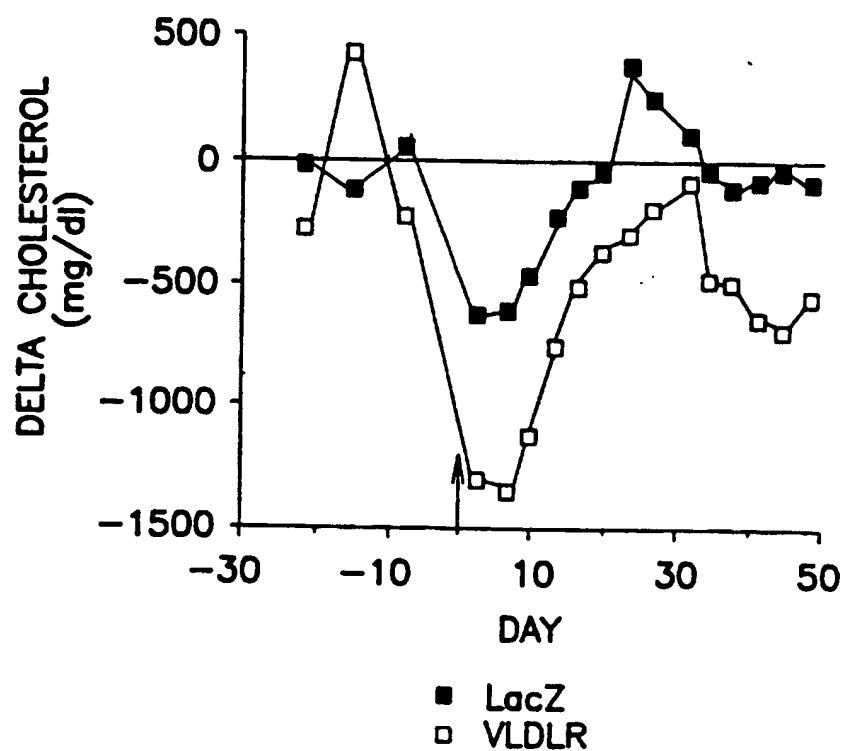


FIG. 7B

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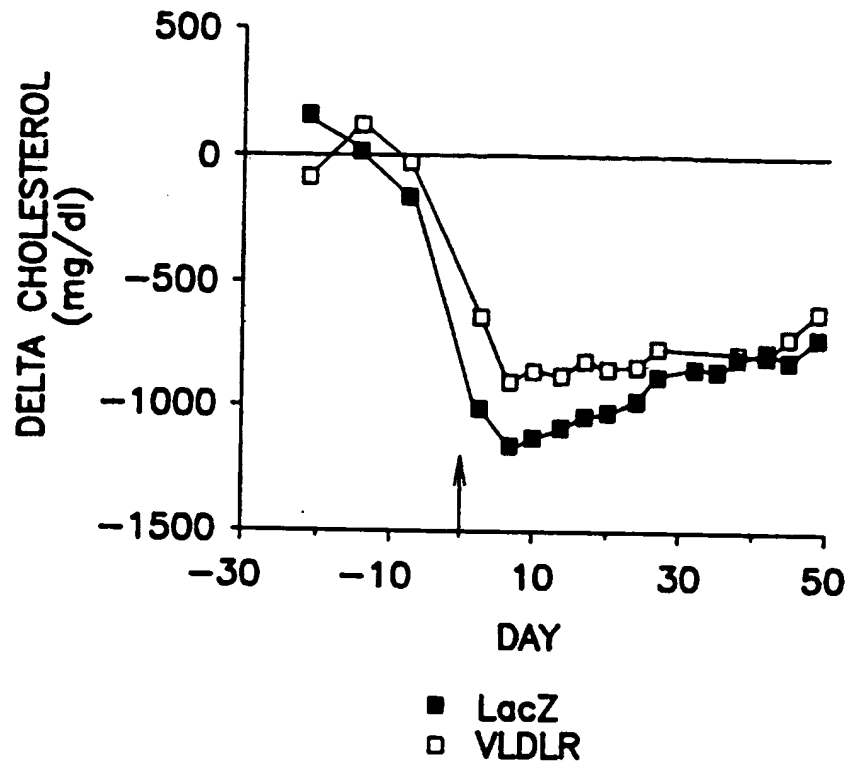


FIG. 7C

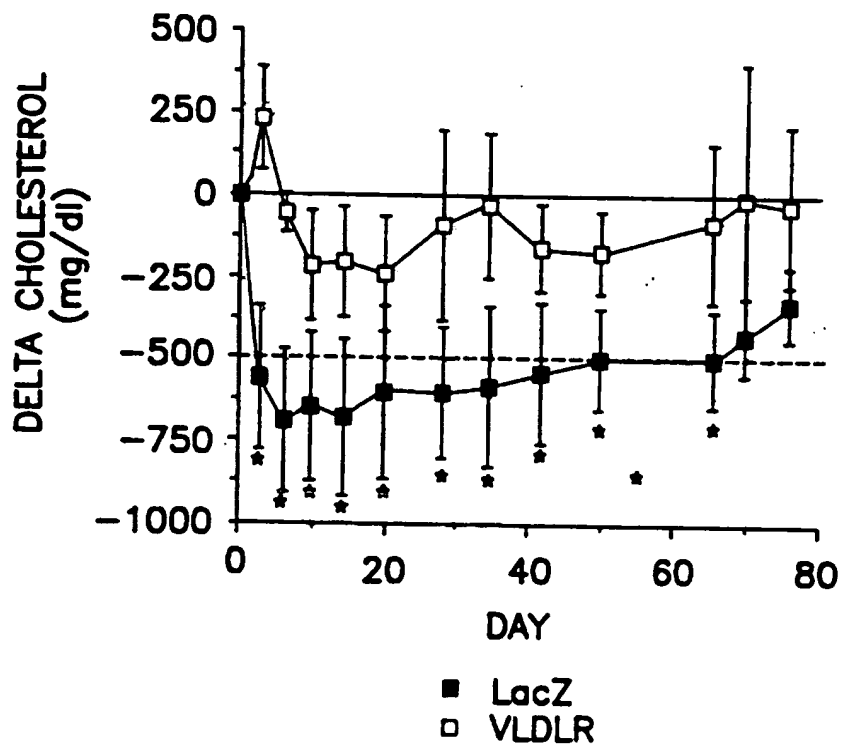


FIG. 7D

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FIGURE 8A

CTCTGCGGGC CGCGGGTGCG GGTCGTCGCT ACCGGCTCTC TCCGTTCTGT	50
GCTCTCTTCT GCTCTCGGCT CCCCACCCCC TCTCCCTTCC CTCCTCTCCC	100
CTTGCCTCCC CTCCTCTGCA GCGCCTGCAT TATTTTCTGC CCGCAGCTCG	150
GCTTGCACTG CTGCTGCAGC CCGGGGAGGT GGCTGGGTGG GTGGGGAGGA	200
GACTGTGCAA GTTGTAGGGG AGGGGGTGCC CTCTTCTTCC CCGCTCCCTT	250
CCCCAGCCAA GTGGTTCCCC TCCTTCTCCC CCTTTCCCCT CCCAGCCCCC	300
ACCTTCTTCC TCTTTCGGAA GGGCTGGTAA CTTGTGCTGC GGAGCGAACG	350
GCGGCGGCGG CGGCGGCGGC GGCACCATCC AGGCGGGCAC C ATG GGC ACG	400
Met Gly Thr	
1	
TCC GCG CTC TGG GCC GTC TGG CTG CTG CTC GCG CTG TGC TGG	442
Ser Ala Leu Trp Ala Val Trp Leu Leu Leu Ala Leu Cys Trp	
5 10 15	
GCG CCC CGG GAG AGC GGC GCC ACC GGA ACC GGG AGA AAA GCC	484
Ala Pro Arg Glu Ser Gly Ala Thr Gly Thr Gly Arg Lys Ala	
20 25 30	
AAA TGT GAA CCC TCC CAA TTC CAG TGC ACA AAT GGT CGC TGT	526
Lys Cys Glu Pro Ser Gln Phe Gln Cys Thr Asn Gly Arg Cys	
35 40 45	
ATT ACG CTG TTG TGG AAA TGT GAT GGG GAT GAA GAC TGT GTT	568
Ile Thr Leu Leu Trp Lys Cys Asp Gly Asp Glu Asp Cys Val	
50 55	
GAC GGC AGT GAT GAA AAG AAC TGT GTA AAG AAG ACG TGT GCT	610
Asp Gly Ser Asp Glu Lys Asn Cys Val Lys Lys Thr Cys Ala	
60 65 70	
GAA TCT GAC TTC GTG TGC AAC AAT GGC CAG TGT GTT CCC AGC	652
Glu Ser Asp Phe Val Cys Asn Asn Gly Gln Cys Val Pro Ser	
75 80 85	
CGA TGG AAG TGT GAT GGA GAT CCT GAC TGC GAA GAT GGT TCA	694
Arg Trp Lys Cys Asp Gly Asp Pro Asp Cys Glu Asp Gly Ser	
90 95 100	
GAT GAA AGC CCA GAA CAG TGC CAT ATG AGA ACA TGC CGC ATA	736
Asp Glu Ser Pro Glu Gln Cys His Met Arg Thr Cys Arg Ile	
105 110 115	
CAT GAA ATC AGC TGT GGC GCC CAT TCT ACT CAG TGT ATC CCA	778
His Glu Ile Ser Cys Gly Ala His Ser Thr Gln Cys Ile Pro	
120 125	

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FIGURE 8B

GTG	TCC	TGG	AGA	TGT	GAT	GGT	GAA	AAT	GAT	TGT	GAC	AGT	GGA	820
Val	Ser	Trp	Arg	Cys	Asp	Gly	Glu	Asn	Asp	Cys	Asp	Ser	Gly	
130					135					140				
GAA	GAT	GAA	GAA	AAC	TGT	GGC	AAT	ATA	ACA	TGT	AGT	CCC	GAC	862
Glu	Asp	Glu	Glu	Asn	Cys	Gly	Asn	Ile	Thr	Cys	Ser	Pro	Asp	
145						150					155			
GAG	TTC	ACC	TGC	TCC	AGT	GGC	CGC	TGC	ATC	TCC	AGG	AAC	TTT	904
Glu	Phe	Thr	Cys	Ser	Ser	Gly	Arg	Cys	Ile	Ser	Arg	Asn	Phe	
		160					165					170		
GTA	TGC	AAT	GGC	CAG	GAT	GAC	TGC	AGC	GAT	GGC	AGT	GAT	GAG	946
Val	Cys	Asn	Gly	Gln	Asp	Asp	Cys	Ser	Asp	Gly	Ser	Asp	Glu	
			175					180					185	
CTG	GAC	TGT	GCC	CCG	CCA	ACC	TGT	GGC	GCC	CAT	GAG	TTC	CAG	988
Leu	Asp	Cys	Ala	Pro	Pro	Thr	Cys	Gly	Ala	His	Glu	Phe	Gln	
				190					195					
TGC	AGC	ACC	TCC	TCC	TGC	ATC	CCC	ATC	AGC	TGG	GTA	TGC	GAC	1030
Cys	Ser	Thr	Ser	Ser	Cys	Ile	Pro	Ile	Ser	Trp	Val	Cys	Asp	
200					205					210				
GAT	GAT	GCA	GAC	TGC	TCC	GAC	CAA	TCT	GAT	GAG	TCC	CTG	GAG	1072
Asp	Asp	Ala	Asp	Cys	Ser	Asp	Gln	Ser	Asp	Glu	Ser	Leu	Glu	
215						220					225			
CAG	TGT	GGC	CGT	CAG	CCA	GTC	ATA	CAC	ACC	AAG	TGT	CCA	GCC	1114
Gln	Cys	Gly	Arg	Gln	Pro	Val	Ile	His	Thr	Lys	Cys	Pro	Ala	
		230					235					240		
AGC	GAA	ATC	CAG	TGC	GGC	TCT	GGC	GAG	TGC	ATC	CAT	AAG	AAG	1156
Ser	Glu	Ile	Gln	Cys	Gly	Ser	Gly	Glu	Cys	Ile	His	Lys	Lys	
			245				250						255	
TGG	CGA	TGT	GAT	GGG	GAC	CCT	GAC	TGC	AAG	GAT	GGC	AGT	GAT	1198
Trp	Arg	Cys	Asp	Gly	Asp	Pro	Asp	Cys	Lys	Asp	Gly	Ser	Asp	
				260					265					
GAG	GTC	AAC	TGT	CCC	TCT	CGA	ACT	TGC	CGA	CCT	GAC	CAA	TTT	1240
Glu	Val	Asn	Cys	Pro	Ser	Arg	Thr	Cys	Arg	Pro	Asp	Gln	Phe	
270					275					280				
GAA	TGT	GAG	GAT	GGC	AGC	TGC	ATC	CAT	GGC	AGC	AGG	CAG	TGT	1282
Glu	Cys	Glu	Asp	Gly	Ser	Cys	Ile	His	Gly	Ser	Arg	Gln	Cys	
285						290					295			
AAT	GGT	ATC	CGA	GAC	TGT	GTC	GAT	GGT	TCC	GAT	GAA	GTC	AAC	1324
Asn	Gly	Ile	Arg	Asp	Cys	Val	Asp	Gly	Ser	Asp	Glu	Val	Asn	
		300					305					310		

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FIGURE 8C

TGC AAA AAT GTC AAT CAG TGC TTG GGC CCT GGA AAA TTC AAG	1366
Cys Lys Asn Val Asn Gln Cys Leu Gly Pro Gly Lys Phe Lys	
315 320 325	
TGC AGA AGT GGA GAA TGC ATA GAT ATC AGC AAA GTA TGT AAC	1408
Cys Arg Ser Gly Glu Cys Ile Asp Ile Ser Lys Val Cys Asn	
330 335	
CAG GAG CAG GAC TGC AGG GAC TGG AGT GAT GAG CCC CTG AAA	1450
Gln Glu Gln Asp Cys Arg Asp Trp Ser Asp Glu Pro Leu Lys	
340 345 350	
GAG TGT CAT ATA AAC GAA TGC TTG GTA AAT AAT GGT GGA TGT	1492
Glu Cys His Ile Asn Glu Cys Leu Val Asn Asn Gly Gly Cys	
355 360 365	
TCT CAT ATC TGC AAA GAC CTA GTT ATA GGC TAC GAG TGT GAC	1534
Ser His Ile Cys Lys Asp Leu Val Ile Gly Tyr Glu Cys Asp	
370 375 380	
TGT GCA GCT GGG TTT GAA CTG ATA GAT AGG AAA ACC TGT GGA	1576
Cys Ala Ala Gly Phe Glu Leu Ile Asp Arg Lys Thr Cys Gly	
385 390 395	
GAT ATT GAT GAA TGC CAA AAT CCA GGA ATC TGC AGT CAA ATT	1618
Asp Ile Asp Glu Cys Gln Asn Pro Gly Ile Cys Ser Gln Ile	
400 405	
TGT ATC AAC TTA AAA GGC GGT TAC AAG TGT GAA TGT AGT CGT	1660
Cys Ile Asn Leu Lys Gly Gly Tyr Lys Cys Glu Cys Ser Arg	
410 415 420	
GCC TAT CAA ATG GAT CTT GCT ACT GGC GTG TGC AAG GCA GTA	1702
Ala Tyr Gln Met Asp Leu Ala Thr Gly Val Cys Lys Ala Val	
425 430 435	
GGC AAA GAG CCA AGT CTG ATC TTC ACT AAT CGA AGA GAC ATC	1744
Gly Lys Glu Pro Ser Leu Ile Phe Thr Asn Arg Arg Asp Ile	
440 445 450	
AGG AAG ATT GGC TTA GAG AGG AAA GAA TAT ATC CAA CTA GTT	1786
Arg Lys Ile Gly Leu Glu Arg Lys Glu Tyr Ile Gln Leu Val	
455 460 465	
GAA CAG CTA AGA AAC ACT GTG GCT CTC GAT GCT GAC ATT GCT	1828
Glu Gln Leu Arg Asn Thr Val Ala Leu Asp Ala Asp Ile Ala	
470 475	
GCC CAG AAA CTA TTC TGG GCC GAT CTA AGC CAA AAG GCT ATC	1870
Ala Gln Lys Leu Phe Trp Ala Asp Leu Ser Gln Lys Ala Ile	
480 485 490	

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FIGURE 8D

TTC	AGT	GCC	TCA	ATT	GAT	GAC	AAG	GTT	GGT	AGA	CAT	GTT	AAA	1912
Phe	Ser	Ala	Ser	Ile	Asp	Asp	Lys	Val	Gly	Arg	His	Val	Lys	
495						500					505			
ATG	ATC	GAC	AAT	GTC	TAT	AAT	CCT	GCA	GCC	ATT	GCT	GTT	GAT	1954
Met	Ile	Asp	Asn	Val	Tyr	Asn	Pro	Ala	Ala	Ile	Ala	Val	Asp	
		510					515					520		
TGG	GTG	TAC	AAG	ACC	ATC	TAC	TGG	ACT	GAT	GCG	GCT	TCT	AAG	1996
Trp	Val	Tyr	Lys	Thr	Ile	Tyr	Trp	Thr	Asp	Ala	Ala	Ser	Lys	
			525					530					535	
ACT	ATT	TCA	GTA	GCT	ACC	CTA	GAT	GGA	ACC	AAG	AGG	AAG	TTC	2038
Thr	Ile	Ser	Val	Ala	Thr	Leu	Asp	Gly	Thr	Lys	Arg	Lys	Phe	
				540					545					
CTG	TTT	AAC	TCT	GAC	TTG	CGA	GAG	CCT	GCC	TCC	ATA	GCT	GTG	2080
Leu	Phe	Asn	Ser	Asp	Leu	Arg	Glu	Pro	Ala	Ser	Ile	Ala	Val	
550					555					560				
GAC	CCA	CTG	TCT	GGC	TTT	GTT	TAC	TGG	TCA	GAC	TGG	GGT	GAA	2122
Asp	Pro	Leu	Ser	Gly	Phe	Val	Tyr	Trp	Ser	Asp	Trp	Gly	Glu	
	565					570					575			
CCA	GCT	AAA	ATA	GAA	AAA	GCA	GGA	ATG	AAT	GGA	TTC	GAT	AGA	2164
Pro	Ala	Lys	Ile	Glu	Lys	Ala	Gly	Met	Asn	Gly	Phe	Asp	Arg	
		580					585					590		
CGT	CCA	CTG	GTG	ACA	GCG	GAT	ATC	CAG	TGG	CCT	AAC	GGA	ATT	2206
Arg	Pro	Leu	Val	Thr	Ala	Asp	Ile	Gln	Trp	Pro	Asn	Gly	Ile	
			595					600					605	
ACA	CTT	GAC	CTT	ATA	AAA	AGT	CGC	CTC	TAT	TGG	CTT	GAT	TCT	2248
Thr	Leu	Asp	Leu	Ile	Lys	Ser	Arg	Leu	Tyr	Trp	Leu	Asp	Ser	
				610					615					
AAG	TTG	CAC	ATG	TTA	TCC	AGC	GTG	GAC	TTG	AAT	GGC	CAA	GAT	2290
Lys	Leu	His	Met	Leu	Ser	Ser	Val	Asp	Leu	Asn	Gly	Gln	Asp	
620					625					630				
CGT	AGG	ATA	GTA	CTA	AAG	TCT	CTG	GAG	TTC	CTA	GCT	CAT	CCT	2332
Arg	Arg	Ile	Val	Leu	Lys	Ser	Leu	Glu	Phe	Leu	Ala	His	Pro	
	635					640					645			
CTT	GCA	CTA	ACA	ATA	TTT	GAG	GAT	CGT	GTC	TAC	TGG	ATA	GAT	2374
Leu	Ala	Leu	Thr	Ile	Phe	Glu	Asp	Arg	Val	Tyr	Trp	Ile	Asp	
		650					655					660		
GGG	GAA	AAT	GAA	GCA	GTC	TAT	GGT	GCC	AAT	AAA	TTC	ACT	GGA	2416
Gly	Glu	Asn	Glu	Ala	Val	Tyr	Gly	Ala	Asn	Lys	Phe	Thr	Gly	
			665					670					675	

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FIGURE 8E

TCA	GAG	CAT	GCC	ACT	CTA	GTC	AAC	AAC	CTG	AAT	GAT	GCC	CAA	2458
Ser	Glu	His	Ala	Thr	Leu	Val	Asn	Asn	Leu	Asn	Asp	Ala	Gln	
				680					685					
GAC	ATC	ATT	GTC	TAT	CAT	GAA	CTT	GTA	CAG	CCA	TCA	GGT	AAA	2500
Asp	Ile	Ile	Val	Tyr	His	Glu	Leu	Val	Gln	Pro	Ser	Gly	Lys	
690					695				700					
AAT	TGG	TGT	GAA	GAA	GAC	ATG	GAG	AAT	GGA	GGA	TGT	GAA	TAC	2545
Asn	Trp	Cys	Glu	Glu	Asp	Met	Glu	Asn	Gly	Gly	Cys	Glu	Tyr	
705						710					715			
CTA	TGC	CTG	CCA	GCA	CCA	CAG	ATT	AAT	GAT	CAC	TCT	CCA	AAA	2584
Leu	Cys	Leu	Pro	Ala	Pro	Gln	Ile	Asn	Asp	His	Ser	Pro	Lys	
		720				725						730		
TAT	ACC	TGT	TCC	TGT	CCC	AGT	GGG	TAC	AAT	GTA	GAG	GAA	AAT	2626
Tyr	Thr	Cys	Ser	Cys	Pro	Ser	Gly	Tyr	Asn	Val	Glu	Glu	Asn	
			735					740					745	
GGC	CGA	GAC	TGT	CAA	AGT	ACT	GCA	ACT	ACT	GTG	ACT	TAC	AGT	2668
Gly	Arg	Asp	Cys	Gln	Ser	Thr	Ala	Thr	Thr	Val	Thr	Tyr	Ser	
				750					755					
GAG	ACA	AAA	GAT	ACG	AAC	ACA	ACA	GAA	ATT	TCA	GCA	ACT	AGT	2710
Glu	Thr	Lys	Asp	Thr	Asn	Thr	Thr	Glu	Ile	Ser	Ala	Thr	Ser	
760					765					770				
GGA	CTA	GTT	CCT	GGA	GGG	ATC	AAT	GTG	ACC	ACA	GCA	GTA	TCA	2752
Gly	Leu	Val	Pro	Gly	Gly	Ile	Asn	Val	Thr	Thr	Ala	Val	Ser	
775						780					785			
GAG	GTC	AGT	GTT	CCC	CCA	AAA	GGG	ACT	TCT	GCC	GCA	TGG	GCC	2794
Glu	Val	Ser	Val	Pro	Pro	Lys	Gly	Thr	Ser	Ala	Ala	Trp	Ala	
		790					795					800		
ATT	CTT	CCT	CTC	TTG	CTC	TTA	GTG	ATG	GCA	GCA	GTA	GGT	GGC	2836
Ile	Leu	Pro	Leu	Leu	Leu	Leu	Val	Met	Ala	Ala	Val	Gly	Gly	
			805					810					815	
TAC	TTG	ATG	TGG	CGG	AAT	TGG	CAA	CAC	AAG	AAC	ATG	AAA	AGC	2878
Tyr	Leu	Met	Trp	Arg	Asn	Trp	Gln	His	Lys	Asn	Met	Lys	Ser	
				820					825					
ATG	AAC	TTT	GAC	AAT	CCT	GTG	TAC	TTG	AAA	ACC	ACT	GAA	GAG	2920
Met	Asn	Phe	Asp	Asn	Pro	Val	Tyr	Leu	Lys	Thr	Thr	Glu	Glu	
830					835					840				
GAC	CTC	TCC	ATA	GAC	ATT	GGT	AGA	CAC	AGT	GCT	TCT	GTT	GGA	2962
Asp	Leu	Ser	Ile	Asp	Ile	Gly	Arg	His	Ser	Ala	Ser	Val	Gly	
	845					850					855			

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FIGURE 8F

CAC ACG TAC CCA GCA ATA TCA GTT GTA AGC ACA GAT GAT GAT	3004
His Thr Tyr Pro Ala Ile Ser Val Val Ser Thr Asp Asp Asp	
860 865 870	
CTA GCT TGACTTCTGT GACAAATGTT GACCTTTGAG GTCTAAACAA	3050
Leu Ala	
ATAATACCCC CGTCGGAATG GTAACCGAGC CAGCAGCTGA AGTCTCTTTT	3100
TCTTCCTCTC GGCTGGAAGA ACATCAAGAT ACCTTTGCGT GGATCAAGCT	3150
TGCTGTACTT GACCGTTTTT ATATTACTTT TGTAATATT CTTGTCCACA	3200
TTCTACTTCA GCTTTGGATG TGGTTACCGA GTATCTGTAA CCCTTGAATT	3250
TCTAGACAGT ATTGCCACCT CTGGCCAAAT ATGCACTTTC CCTAGAAAGC	3300
CATATTCCAG CAGTGAAACT TGTGCTATAG TGTATACCAC CTGTACATAC	3350
ATTGTATAGG CCATCTGTAA ATATCCCAGA GAACAATCAC TATTCTTAAG	3400
CACTTTGAAA ATATTTCTAT GTAAATTATT GTAAACTTTT TCAATGGTTG	3450
GGACAATGGC AATAGGACAA AACGGGTTAC TAAGATGAAA TTGCCAAAAA	3500
AATTTATAAA CTAATTTTGG TACGTATGAA TGATATCTTT GACCTCAATG	3550
GAGGTTTGCA AAGACTGAGT GTTCAAATA CTGTACATTT TTTTTCAGT	3600
GCTAAAAAAT TAAACCAAGC AGCTTAAAAA AAAAAAAAAA AAAAAAAAAA	3650
AAAAAA	3656

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FIGURE 9A

GAATTCGCTA GCATCATCAA TAATATACCT TATTTTGGAT TGAAGCCAAT ATGATAATGA
60

GGGGGTGGAG TTTGTGACGT GCGCGGGGC GTGGGAACGG GCGGGGTGAC GTAGTAGTGT
120

GGCGGAAGTG TGATGTTGCA AGTGTGGCGG AACACATGTA AGCGACGGAT GTGGCAAAAG
180

TGACGTTTTT GGTGTGCGCC GGTGTACACA GGAAGTGACA ATTTTCGCGC GGTTTTAGGC
240

GGATGTTGTA GTAAATTTGG GCGTAACCGA GTAAGATTTG GCCATTTTCG CGGGAAAAC
300

GAATAAGAGG AAGTGAAATC TGAATAATTT TGTGTTACTC ATAGCGCGTA ATATTTGTCT
360

AGGGAGATCA GCCTGCAGGT CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG
420

CCCAACGACC CCCGCCCATTT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA
480

GGGACTTTCC ATTGACGTCA ATGGGTGGAG TATTTACGGT AAAGTGCCCA CTTGGCAGTA
540

CATCAAGTGT ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC
600

GCCTGGCATT ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC
660

GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA
720

TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG
780

TTTTGGCACC AAAATCAACG GGAAGTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG
840

CAAATGGGCG GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT
900

AGAGAACCCA CTGCTTAACT GGCTTATCGA AATTAATACG ACTCACTATA GGGAGACCCA
960

AGCTTCTCTG CGGGCCGCGG GTGCGGGTCG TCGCTACCGG CTCTCTCCGT TCTGTGCTCT
1020

CTTCTGCTCT CGGCTCCCCA CCCCTCTCC CTTCCCTCCT CTCCCCTTGC CTCCCCTCCT
1080

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FIGURE 9B

CTGCAGCGCC TGCATTATTT TCTGCCCCGCA GCTCGGCTTG CACTGCTGCT GCAGCCCGGG
1140

GAGGTGGCTG GGTGGGTGGG GAGGAGACTG TGCAAGTTGT AGGGGAGGGG GTGCCCTCTT
1200

CTTCCCCGCT CCCTTCCCCA GCCAAGTGGT TCCCCTCCTT CTCCCCCTTT CCCCTCCCAG
1260

CCCCACCTT CTTCTCTTT CGGAAGGGCT GGTAAGTTGT CGTGCGGAGC GAACGGCGGC
1320

GGCGGCGGCG GCGGCGGCAC CATCCAGGCG GGCACCATGG GCACGTCCGC GCTCTGGGCC
1380

GTCTGGCTGC TGCTCGCGCT GTGCTGGGCG CCCCAGGAGA GCGGCGCCAC CGGAACCGGG
1440

AGAAAAGCCA AATGTGAACC CTCCCAATTC CAGTGCACAA ATGGTCGCTG TATTACGCTG
1500

TTGTGGAAAT GTGATGGGGA TGAAGACTGT GTTGACGGCA GTGATGAAAA GAACTGTGTA
1560

AAGAAGACGT GTGCTGAATC TGAATTCTG TGCAACAATG GCCAGTGTGT TCCAGCCGA
1620

TGGAAGTGTG ATGGAGATCC TGAATGCGAA GATGGTTCAG ATGAAAGCCC AGAACAGTGC
1680

CATATGAGAA CATGCCGCAT ACATGAAATC AGCTGTGGCG CCCATTCTAC TCAGTGTATC
1740

CCAGTGTCTT GGAGATGTGA TGGTGAAAAT GATTGTGACA GTGGAGAAGA TGAAGAAAAC
1800

TGTGGCAATA TAACATGTAG TCCCGACGAG TTCACCTGCT CCAGTGGCCG CTGCATCTCC
1860

AGGAACTTTG TATGCAATGG CCAGGATGAC TGCAGCGATG GCAGTGATGA GCTGGACTGT
1920

GCCCCGCCAA CCTGTGGCGC CCATGAGTTC CAGTGCAGCA CCTCCTCCTG CATCCCCATC
1980

AGCTGGGTAT GCGACGATGA TGCAGACTGC TCCGACCAAT CTGATGAGTC CCTGGAGCAG
2040

TGTGGCCGTC AGCCAGTCAT ACACACCAAG TGTCCAGCCA GCGAAATCCA GTGCGGCTCT
2100

GGCGAGTGCA TCCATAAGAA GTGGCGATGT GATGGGGACC CTGACTGCAA GGATGGCAGT
2160

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FIGURE 9C

GATGAGGTCA ACTGTCCCTC TCGAACTTGC CGACCTGACC AATTTGAATG TGAGGATGGC
2220

AGCTGCATCC ATGGCAGCAG GCAGTGTAAT GGTATCCGAG ACTGTGTCGA TGGTTCCGAT
2280

GAAGTCAACT GCAAAAATGT CAATCAGTGC TTGGGCCCTG GAAAATTCAA GTGCAGAAGT
2340

GGAGAATGCA TAGATATCAG CAAAGTATGT AACCAGGAGC AGGACTGCAG GGACTGGAGT
2400

GATGAGCCCC TGAAAGAGTG TCATATAAAC GAATGCTTGG TAAATAATGG TGGATGTTCT
2460

CATATCTGCA AAGACCTAGT TATAGGCTAC GAGTGTGACT GTGCAGCTGG GTTTGAACTG
2520

ATAGATAGGA AAACCTGTGG AGATATTGAT GAATGCCAAA ATCCAGGAAT CTGCAGTCAA
2580

ATTTGTATCA ACTTAAAAGG CGGTTACAAG TGTGAATGTA GTCGTGCCTA TCAAATGGAT
2640

CTTGCTACTG GCGTGTGCAA GGCAGTAGGC AAAGAGCCAA GTCTGATCTT CACTAATCGA
2700

AGAGACATCA GGAAGATTGG CTTAGAGAGG AAAGAATATA TCCAACCTAGT TGAACAGCTA
2760

AGAAACACTG TGGCTCTCGA TGCTGACATT GCTGCCCAGA AACTATTCTG GGCCGATCTA
2820

AGCCAAAAGG CTATCTTCAG TGCCTCAATT GATGACAAGG TTGGTAGACA TGTTAAAATG
2880

ATCGACAATG TCTATAATCC TGCAGCCATT GCTGTTGATT GGGTGTACAA GACCATCTAC
2940

TGGACTGATG CGGCTTCTAA GACTATTTCA GTAGCTACCC TAGATGGAAC CAAGAGGAAG
3000

TTCCTGTTTA ACTCTGACTT GCGAGAGCCT GCCTCCATAG CTGTGGACCC ACTGTCTGGC
3060

TTTGTTTACT GGTCAGACTG GGGTGAACCA GCTAAAATAG AAAAAGCAGG AATGAATGGA
3120

TTCGATAGAC GTCCACTGGT GACAGCGGAT ATCCAGTGGC CTAACGGAAT TACACTTGAC
3180

CTTATAAAAA GTCGCCTCTA TTGGCTTGAT TCTAAGTTGC ACATGTTATC CAGCGTGGAC
3240

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FIGURE 9D

TTGAATGGCC AAGATCGTAG GATAGTACTA AAGTCTCTGG AGTTCCTAGC TCATCCTCTT
3300

GCACTAACAA TATTTGAGGA TCGTGTCTAC TGGATAGATG GGGAAAATGA AGCAGTCTAT
3360

GGTGCCAATA AATTCACTGG ATCAGAGCAT GCCACTCTAG TCAACAACCT GAATGATGCC
3420

CAAGACATCA TTGTCTATCA TGAACCTGTA CAGCCATCAG GTAAAAATTG GTGTGAAGAA
3480

GACATGGAGA ATGGAGGATG TGAATACCTA TGCCTGCCAG CACCACAGAT TAATGATCAC
3540

TCTCCAAAAT ATACCTGTTC CTGTCCCAGT GGGTACAATG TAGAGGAAAA TGGCCGAGAC
3600

TGTCAAAGTA CTGCAACTAC TGTGACTTAG AGACAAAAGA TACGAACACA ACAGAAATTT
3660

CAGCAACTAG TGGACTAGTT CCTGGAGGGA TCAATGTGAC CACAGCAGTA TCAGAGGTCA
3720

GTGTTCCCCC AAAAGGGACT TCTGCCGCAT GGGCCATTCT TCCTCTCTTG CTCTTAGTGA
3780

TGGCAGCAGT AGGTGGCTAC TTGATGTGGC GGAATTGGCA ACACAAGAAC ATGAAAAGCA
3840

TGAACCTTGA CAATCCTGTG TACTTGAAAA CCACTGAAGA GGACCTCTCC ATAGACATTG
3900

GTAGACACAG TGCTTCTGTT GGACACACGT ACCCAGCAAT ATCAGTTGTA AGCACAGATG
3960

ATGATCTAGC TTGACTTCTG TGACAAATGT TGACCTTTGA GGTCTAAACA AATAATACCC
4020

CCGTCGGAAT GGTAACCGAG CCAGCAGCTG AAGTCTCTTT TTCTTCCTCT CGGCTGGAAG
4080

AACATCAAGA TACCTTTGCG TGGATCAAGC TTGGTACCGA GCTCGGATCC ACTAGTAACG
4140

GCCGCCAGTG TGCTGGAATT CTGCAGATAT CCATCACACT GGC GGCCGCG GGGATCCAGA
4200

CATGATAAGA TACATTGATG AGTTTGGACA AACCACAACT AGAATGCAGT GAAAAAATG
4260

CTTTATTTGT GAAATTTGTG ATGCTATTGC TTTATTTGTA ACCATTATAA GCTGCAATAA
4320

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FIGURE 9E

ACAAGTTAAC AACACAATT GCATTCATTT TATGTTTCAG GTTCAGGGGG AGGTGTGGGA
4380

GGTTTTTTCG GATCCTCTAG AGTCGACCTG CAGGCTGATC TGGAAGGTGC TGAGGTACGA
4440

TGAGACCCGC ACCAGGTGCA GACCCTGCGA GTGTGGCGGT AAACATATTA GGAACCAGCC
4500

TGTGATGCTG GATGTGACCG AGGAGCTGAG GCCCGATCAC TTGGTGCTGG CCTGCACCCC
4560

CGCTGAGTTT GGCTCTAGCG ATGAAGATAC AGATTGAGGT ACTGAAATGT GTGGGCGTGG
4620

CTTAAGGGTG GGAAAGAATA TATAAGGTGG GGGTCTTATG TAGTTTTGTA TCTGTTTTGC
4680

AGCAGCCGCC GCCGCCATGA GCACCAACTC GTTTGATGGA AGCATTGTGA GCTCATATTT
4740

GACAACGCGC ATGCCCCCAT GGGCCGGGGT GCGTCAGAAT GTGATGGGCT CCAGCATTTGA
4800

TGGTCGCCCC GTCCTGCCCC CAAACTCTAC TACCTTGACC TACGAGACCG TGTCTGGAAC
4860

GCCGTTGGAG ACTGCAGCCT CCGCCGCCGC TTCAGCCGCT GCAGCCACCG CCCGCGGGAT
4920

TGTGACTGAC TTTGCTTTCC TGAGCCCGCT TGCAAGCAGT GCAGCTTCCC GTTCATCCGC
4980

CCGCGATGAC AAGTTGACGG CTCTTTTGGC ACAATTGGAT TCTTTGACCC GGGAACTTAA
5040

TGTCGTTTCT CAGCAGCTGT TGGATCTGCG CCAGCAGGTT TCTGCCCTGA AGGCTTCCTC
5100

CCCTCCCAAT GCGGTTTAAA ACATAAATAA AAAACCAGAC TCTGTTTGGA TTTGGATCAA
5160

GCAAGTGTCT TGCTGTCTTT ATTTAGGGGT TTTGCGCGCG CGGTAGGCCC GGGACCAGCG
5220

GTCTCGGTCTG TTGAGGGTCC TGTGTATTTT TTCCAGGACG TGGTAAAGGT GACTCTGGAT
5280

GTTCAGATAC ATGGGCATAA GCCCGTCTCT GGGGTGGAGG TAGCACC ACT GCAGAGCTTC
5340

ATGCTGCGGG GTGGTGTGTGT AGATGATCCA GTCGTAGCAG GAGCGCTGGG CGTGGTGCCT
5400

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FIGURE 9F

AAAAATGTCT TTCAGTAGCA AGCTGATTGC CAGGGGCAGG CCCTTGGTGT AAGTGTTTAC
5460

AAAGCGGTTA AGCTGGGATG GGTGCATACG TGGGGATATG AGATGCATCT TGGACTGTAT
5520

TTTtaggttg GCTATGTTCC CAGCCATATC CCTCCGGGGA TTCATGTTGT GCAGAACCAC
5580

CAGCACAGTG TATCCGGTGC ACTTGGGAAA TTTGTCATGT AGCTTAGAAG GAAATGCGTG
5640

GAAGAACTTG GAGACGCCCT TGTGACCTCC AAGATTTTCC ATGCATTCGT CCATAATGAT
5700

GGCAATGGGC CCACGGGCGG CGGCCTGGGC GAAGATATTT CTGGGATCAC TAACGTCATA
5760

GTTGTGTTCC AGGATGAGAT CGTCATAGGC CATTTTTACA AAGCGCGGGC GGAGGGTGCC
5820

AGACTGCGGT ATAATGGTTC CATCCGGCCC AGGGGCGTAG TTACCCTCAC AGATTTGCAT
5880

TTCCCACGCT TTGAGTTCAG ATGGGGGGAT CATGTCTACC TCGGGGCGGA TGAAGAAAAC
5940

GGTTTCCGGG GTAGGGGAGA TCAGCTGGGA AGAAAGCAGG TTCCTGAGCA GCTGCGACTT
6000

ACCGCAGCCG GTGGGCCCCGT AAATCACACC TATTACCGGG TGCAACTGGT AGTTAAGAGA
6060

GCTGCAGCTG CCGTCATCCC TGAGCAGGGG GGCCACTTCG TTAAGCATGT CCCTGACTCG
6120

CATGTTTTCC CTGACCAAAT CCGCCAGAAG GCGCTCGCCG CCCAGCGATA GCAGTTCTTG
6180

CAAGGAAGCA AAGTTTTTCA ACGGTTTGAG ACCGTCCGCC GTAGGCATGC TTTTGAGCGT
6240

TTGACCAAGC AGTTCCAGGC GGTCCCACAG CTCGGTCACC TGCTCTACGG CATCTCGATC
6300

CAGCATATCT CCTCGTTTCG CGGGTTGGGG CGGCTTTCGC TGTACGGCAG TAGTCGGTGC
6360

TCGTCCAGAC GGGCCAGGGT CATGTCCTTC CACGGGCGCA GGGTCCTCGT CAGCGTAGTC
6420

TGGGTCACGG TGAAGGGGTG CGCTCCGGGC TGC GCGCTGG CCAGGGTGCG CTTGAGGCTG
6480

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FIGURE 9G

GTCCTGCTGG TGCTGAAGCG CTGCCGGTCT TCGCCCTGCG CGTCGGCCAG GTAGCATTTG
6540

ACCATGGTGT CATAGTCCAG CCCCTCCGCG GCGTGCCCTT TGGCGCGCAG CTTGCCCTTG
6600

GAGGAGGCGC CGCACGAGGG GCAGTGCAGA CTTTGTAGGG CGTAGAGCTT GGGCGCGAGA
6660

AATACCGATT CCGGGGAGTA GGCATCCGCG CCGCAGGCC CGCAGACGGT CTCGCATTCC
6720

ACGAGCCAGG TGAGCTCTGG CCGTTCGGGG TCAAAAACCA GGTTCCTCCC ATGCTTTTGT
6780

ATGCGTTTCT TACCTCTGGT TTCCATGAGC CCGTGTCCAC GCTCGGTGAC GAAAAGGCTG
6840

TCCGTGTCCC CGTATACAGA CTTGAGAGGC CTGTCCTCGA CCGATGCCCT TGAGAGCCTT
6900

CAACCCAGTC AGCTCCTTCC GGTGGGCGCG GGGCATGACT ATCGTCGCCG CACTTATGAC
6960

TGTCTTCTTT ATCATGCAAC TCGTAGGACA GGTGCCGGCA GCGCTCTGGG TCATTTTCGG
7020

CGAGGACCGC TTTCGCTGGA GCGCGACGAT GATCGGCCTG TCGCTTGCGG TATTCGGAAT
7080

CTTGACGCGC CTCGCTCAAG CCTTCGTCAC TGGTCCCGCC ACCAAACGTT TCGGCGAGAA
7140

GCAGGCCATT ATCGCCGGCA TGGCGGCCGA CGCGCTGGGC TACGTCTTGC TGGCGTTTCG
7200

GACGCGAGGC TGGATGGCCT TCCCCATTAT GATTCTTCTC GCTTCGGGCG GCATCGGGAT
7260

GCCCGCGTTG CAGGCCATGC TGTCCAGGCA GGTAGATGAC GACCATCAGG GACAGCTTCA
7320

AGGATCGCTC GCGGCTCTTA CCAGCCTAAC TTCGATCACT GGACCGCTGA TCGTCACGGC
7380

GATTTATGCC GCCTCGGCGA GCACATGGAA CGGGTTGGCA TGGATTGTAG GCGCCGCCCT
7440

ATACCTTGTC TGCCTCCCCG CGTTGCGTCG CCGTGCATGG AGCCGGGCCA CCTCGACCTG
7500

AATGGAAGCC GCGGGCACCT CGCTAACGGA TTCACCACTC CAAGAATTGG AGCCAATCAA
7560

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FIGURE 9H

TTCTTGCGGA GAACTGTGAA TGCGCAAACC AACCCTTGGC AGAACATATC CATCGCGTCC
7620

GCCATCTCCA GCAGCCGCAC GCGGCGCATC TCGGGCAGCG TTGGGTCCTG GCCACGGGGTG
7680

CGCATGATCG TGCTCCTGTC GTTGAGGACC CGGCTAGGCT GGCGGGGGTTG CCTTACTGGT
7740

TAGCAGAATG AATCACCGAT ACGCGAGCGA ACGTGAAGCG ACTGCTGCTG CAAAACGTCT
7800

GCGACCTGAG CAACAACATG AATGGTCTTC GGTTCCTGTTG TTTCGTAAAG TCTGGAAACG
7860

CGGAAGTCAG CGCCCTGCAC CATTATGTTT CGGATCTGCA TCGCAGGATG CTGCTGGCTA
7920

CCCTGTGGAA CACCTACATC TGTATTAACG AAGCCTTTCT CAATGCTCAC GCTGTAGGTA
7980

TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT GTGCACGAAC CCCCCGTTCA
8040

GCCCGACCGC TCGCCTTAT CCGGTAATA TCGTCTTGAG TCCAACCCGG TAAGACACGA
8100

CTTATCGCCA CTGGCAGCAG CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG
8160

TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA CAGTATTTGG
8220

TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAGA GTTGGTAGCT CTTGATCCGG
8280

CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTGTTGC AAGCAGCAGA TTACGCGCAG
8340

AAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGTGGAA
8400

CGAAACTCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT
8460

CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT AAAGTTGGTC
8520

TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTTGTTTC
8580

ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC
8640

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FIGURE 9I

TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC
8700

AATAAACCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC
8760

CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTTT
8820

GCGCAACGTT GTTGCCATTG CTGCAGGCAT CGTGGTGTC ACGCTCGTCGT TTGGTATGGC
8880

TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA TGATCCCCCA TGTTGTGCAA
8940

AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT
9000

ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG
9060

CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTA TGCGGCGACC
9120

GAGTTGCTCT TGCCCGGCGT CAACACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA
9180

AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAAACTC TCAAGGATCT TACCGCTGTT
9240

GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTTACTTT
9300

CACCAGCGTT TCTGGGTGAG CAAAACAGG AAGGCAAAAT GCCGCAAAA AGGGAATAAG
9360

GGCGACACGG AAATGTTGAA TACTCATACT CTCCTTTTT CAATATTATT GAAGCATTTA
9420

TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT
9480

AGGGGTTCG CGCACATTTT CCCGAAAAGT GCCACCTGAC GTCTAAGAAA CCATTATTAT
9540

CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCC TTTCTGCTTC AA
9592

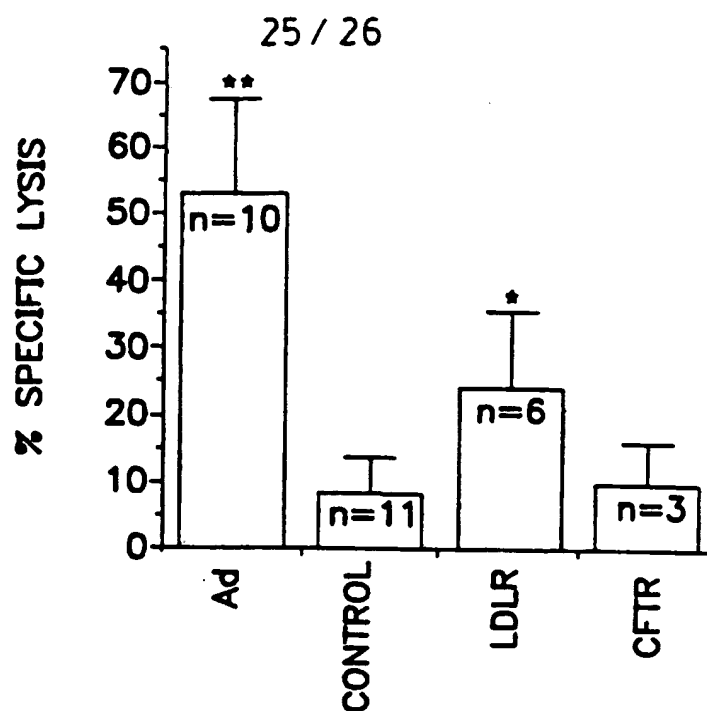


FIG. 10A

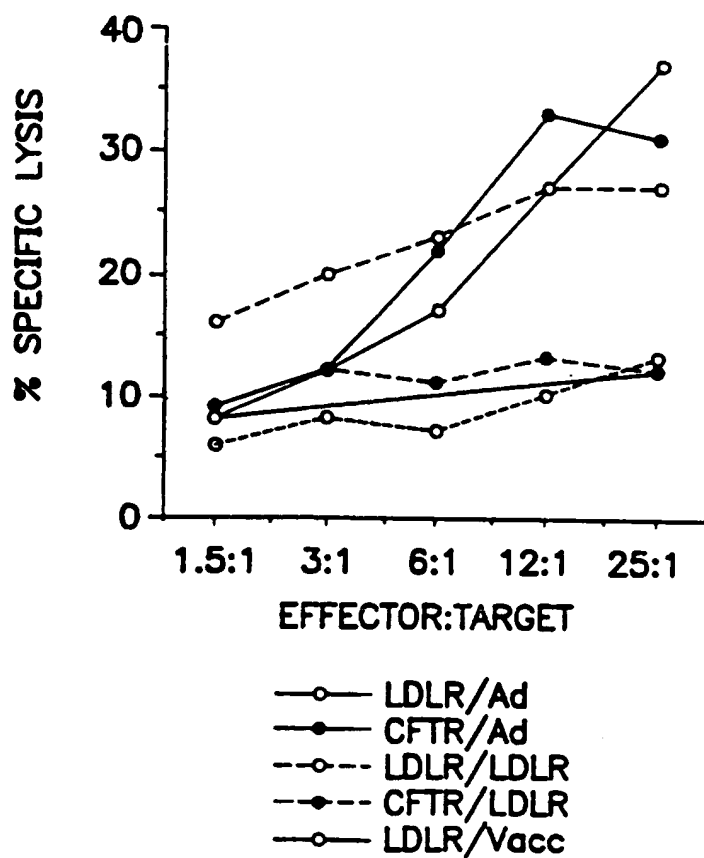


FIG. 10B

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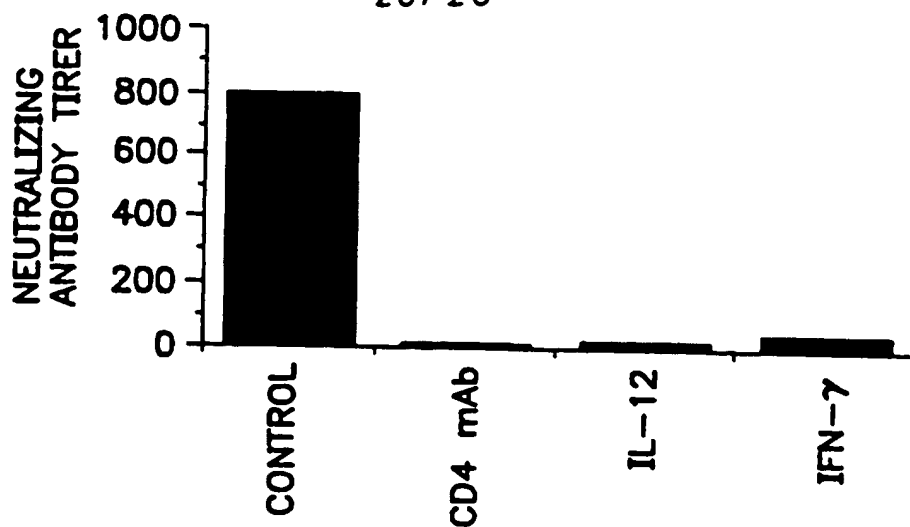


FIG. IIA

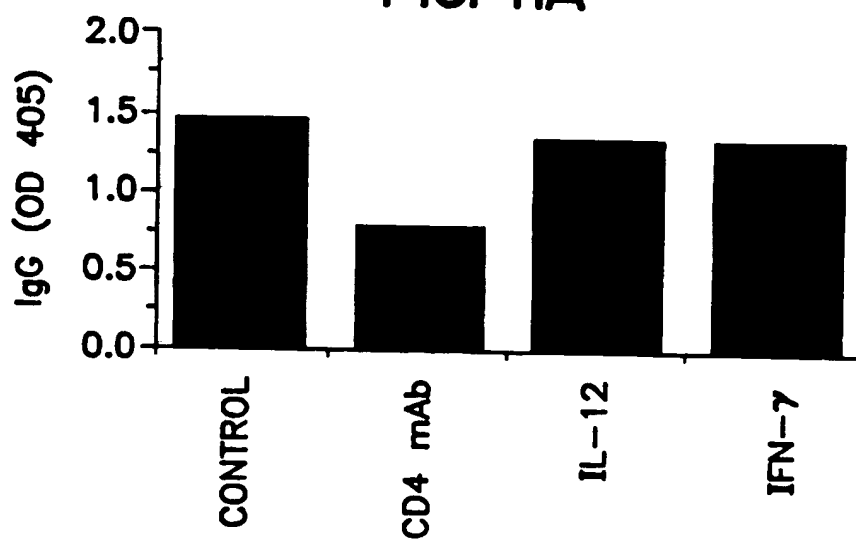


FIG. IIB

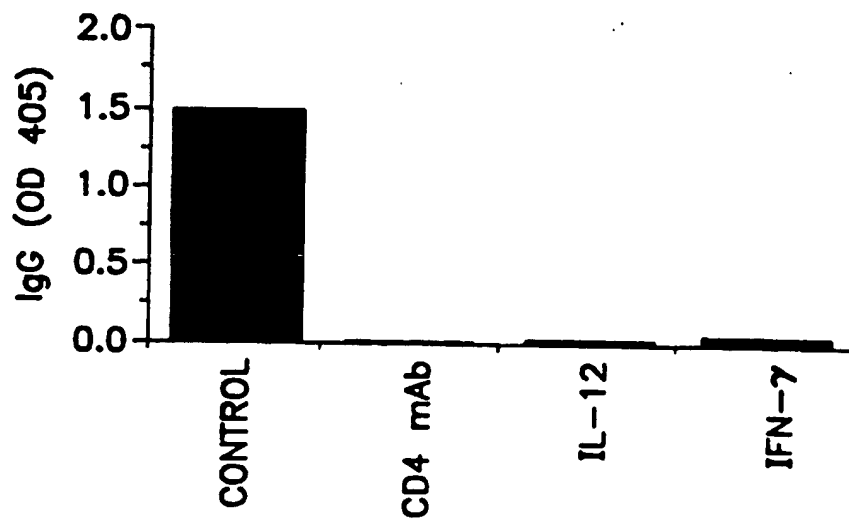


FIG. IIC

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C12N5/10 A61K38/17 //C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 18, 6 May 1994, MD US, pages 13695-13702, XP002007097 K.F.KOZARSKY ET AL.: "In vivo correction of low density lipoprotein receptor deficiency in the Watanabe heritable hyperlipidemic rabbit with recombinant adenoviruses" cited in the application see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-9

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

1 July 1996

Date of mailing of the international search report

25.07.96

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE JOURNAL OF CLINICAL INVESTIGATION, vol. 92, no. 2, August 1993, pages 883-893, XP000574730 S.ISHIBASHI ET AL.: "Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery" cited in the application</p> <p>---</p>	1-9
A	<p>SOMATIC CELL AND MOLECULAR GENETICS, vol. 19, no. 6, November 1993, pages 557-569, XP000574726 M-E-GAFVELS ET AL. : "Cloning of a cDNA encoding a putative human very low density lipoprotein/apolipoprotein E receptor and assignment of the gene to chromosome 9pter-p23" cited in the application see the whole document</p> <p>---</p>	1-9
A	<p>HUMAN MOLECULAR GENETICS, vol. 3, no. 4, April 1994, OXFORD GB, pages 531-537, XP002007098 J.C.WEBB ET AL.: "Characterization and tissue-specific expression of the human 'very low density lipoprotein (VLDL) receptor' mRNA" see the whole document</p> <p>-----</p>	1-9